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(71) Applicant (for all designated States except US): GENEN-TECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): GODDARD, Audrey [CA/US]; 110 Congo Street, San Francisco, CA 94131 (US). GURNEY, Austin, L. [US/US]; 1 Debbie Lane, Belmont, CA 94002 (US). SMITH, Victoria [AU/US]; 19 Dwight Road, Burlingame, CA 94010 (US). HONGO, Jo-Anne, S. [US/US]; 31 Shaw Court, Redwood City, CA 94061 (US). DE SAUVAGE, Frederic [BE/US]; 187 Shooting Star Isle, Foster City, CA 94404 (US).

(74) Agents: CONLEY, Deirdre, L. et al.; c/o Genentech, INC., MS 49, 1 DNA Way, South San Francisco, CA 94080-4990 (US).

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(54) Title: COMPOSITIONS AND METHODS FOR TREATMENT OF CANCER

(57) Abstract: The present invention concerns compositions and methods for the treatment of disorders characterized by the over-expression of an LIV-1. More specifically, the compositions include DNA and amino acid sequences of an LIV-1, antibodies to an LIV-1, and methods for the treatment of a mammal susceptible to or diagnosed with cancer wherein an LIV-1 is overexpressed.

COMPOSITIONS AND METHODS FOR TREATMENT OF CANCER

FIELD OF THE INVENTION

5   The present invention concerns compositions and methods for the treatment of disorders characterized by the overexpression of a LIV-1 gene product in tumors. The compositions comprise a nucleic acid, a polypeptide encoded by the nucleic acid, and a compound, preferably an antibody or fragment thereof, that binds to the polypeptide. preferably binding to the extracellular domain of LIV-1 polypeptide.

BACKGROUND OF THE INVENTION

10   Breast cancer is a common and devastating form of cancer, affecting millions of women per year throughout the world. Many breast tumors are estrogen sensitive and frequently treatable with compounds that interfere with estrogen binding to estrogen receptors (ERs) expressed on the breast tumor tissue. Detecting the level of ER expression and sensitivity to estrogen stimulation is useful for determining that antihormone-type chemotherapy may succeed in a particular patient.

15   The overexpression of estrogen-inducible genes, pLIV-1 and pLIV2 (also designated pS2), occurs in some breast tumors which also express the estrogen receptor. (Manning, D. L., et al.. European J. Cancer 29A(10):1462-1468 (1993): Manning, D.L. et al., *European J. Cancer* 30A(5):675-678 (1994); Manning, D.L., et al., *Acta Oncologica* 34 (5):641-646 (1995); Manning, D.L. et al.. U.S. Patent No. 5.693,465). Expression of pLIV-1, but not pS2, is associated with metastasis of breast cancer cells to regional lymph nodes (Manning et al., USPN

20   5,692,465).

In addition. the pathogenesis of various human malignancies, including breast cancer, is affected by proto-oncogenes that encode growth factors and growth factor receptors. Human ErbB2 gene (*erb*B2, also known as *her2*. or c-*erbB*-2). which encodes a 185-kd transmembrane glycoprotein receptor (ErbB2. also known as HER2 or p185^{HER2}) related to the epidermal growth factor receptor (EGFR), is overexpressed in about 25% to 30% of human

25   breast cancer (Slamon *et al.. Science* 235:177-182 [1987]: Slamon *et al.. Science* 244:707-712 [1989]).

Several lines of evidence support a direct role for ErbB2 in the pathogenesis and clinical aggressiveness of ErbB2-overexpressing tumors. The introduction of ErbB2 into non-neoplastic cells causes their malignant transformation (Hudziak *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7159-7163 [1987]; DiFiore *et al.*, *Science* 237:78-182 [1987]). Transgenic mice that express ErbB2 develop mammary tumors (Guy *et al.*, *Proc. Natl. Acad. Sci. USA*

30   89:10578-10582 [1992]). ErbB2 overexpression is commonly regarded as a predictor of a poor prognosis in humans, especially in patients with primary disease that involves axillary lymph nodes (Slamon *et al.*. [1987] and [1989], *supra*: Ravdin and Chamness, *Gene* 159:19-27 [1995]; and Hynes and Stern. *Biochim Biophys Acta* 1198:165-184 [1994]).

Antibodies directed against human *erb*B2 protein products (anti-ErbB2 antibodies) and against proteins

35   encoded by the rat equivalent of the *erb*B2 gene (*neu*) (anti-*neu* protein antibodies) down-modulate cell surface expression of p185 on B104-1-1 cells (NIH-3T3 cells transfected with the *neu* proto-oncogene) and inhibit colony formation of these cells. Drebin *et al.. Cell* 41:695-706 (1985). Biological effects of anti-*neu* protein antibodies are

reviewed in Myers *et al.*, *Meth. Enzym.* 198:277-290 (1991). See also WO94/22478 published October 13. 1994.

The anti-ErbB2 antibody, 4D5, exhibited anti-proliferative effects on the SKDR3 human breast tumor cell line, inhibiting cellular proliferation by approximately 56%, and sensitizing p185^{erbB2}-overexpressing breast tumor cell lines to the cytotoxic effects of TNF-α. See Hudziak *et al.*, *Mol. Cell. Biol.* 9(3):1165-1172 (1989). See also WO89/06692 published July 27, 1989. The anti-ErbB2 antibodies discussed in Hudziak *et al.* are further characterized in Fendly *et al.* *Cancer Research* 50:1550-1558 (1990); Kotts *et al. In Vitro* 26(3):59A (1990); Sarup *et al. Growth Regulation* 1:72-82 (1991); Shepard *et al. J. Clin. Immunol.* 11(3):117-127 (1991); Kumar *et al. Mol. Cell. Biol.* 11(2):979-986 (1991); Lewis *et al. Cancer Immunol. Immunother.* 37:255-263 (1993); Pietras *et al. Oncogene* 9:1829-1838 (1994); Vitetta *et al. Cancer Research* 54:5301-5309 (1994); Sliwkowski *et al. J. Biol. Chem.* 269(20):14661-14665 (1994); Scott *et al. J. Biol. Chem.* 266:14300-5 (1991); and D'souza *et al. Proc. Natl. Acad. Sci.* 91:7202-7206 (1994).

ErbB2 overexpression is also linked to sensitivity and/or resistance to hormone therapy and chemotherapeutic regimens, including CMF (cyclophosphamide, methotrexate, and fluoruracil) and anthracyclines (Baselga *et al.*, *Oncology* 11(3 Suppl 1):43-48 [1997]). Despite the association of ErbB2 overexpression with poor prognosis, the odds of HER2-positive patients responding clinically to treatment with taxanes were greater than three times those of HER2-negative patients (*Ibid*). rhuMab HER2 was shown to enhance the activity of paclitaxel (TAXOL®) and doxorubicin against breast cancer xenografts in nude mice injected with BT-474 human breast adenocarcinoma cells, which express high levels of HER2 (Baselga *et al.*, *Breast Cancer. Proceedings of ASCO*, Vol. 13, Abstract 53 [1994]).

Because breast and other cancers pose constant threats to health, there is a continuing need to develop treatments for cancers by using methods that target cancer cells without simultaneously harming large numbers of non-cancerous cells, thereby limiting adverse side effects associated with traditional cancer chemotherapy.

SUMMARY OF THE INVENTION

The present invention relates to the discovery of a unique protein, LIV-1-164647, that is overexpressed in some tumor tissues, such as in prostate, colon, lung, breast, and a population of breast tumors that overexpress LIV-1-164647, but do not overexpress ErbB2. The present invention further relates to nucleic acid sequences and amino acid sequences having homology to herein disclosed LIV-1 gene sequence (designated DNA164647) and the amino acid sequence of LIV-1 protein encoded by DNA164647. Applicants' discovery that LIV-1-164647 is overexpressed in tumor cells led to the additional discoveries of compositions for detection and treatment of tumor cells and methods of carrying out such detection and treatment.

In one aspect, the present invention relates to a nucleic acid sequence having homology to the nucleic acid sequence of DNA164647 (SEQ ID NO:3 (coding strand)), or a portion thereof. Preferably the homology is at least approximately 80% homology, more preferably at least approximately 90%, still more preferably at least approximately 95%, and most preferably at least approximately 97% homology. Preferably, the nucleic acid of the invention encodes an aqueous soluble extracellular domain (ECD) that is at least 80% homologous to the DNA164647 (SEQ ID NO:3) from approximately nucleic acid 73 to approximately 1060. Preferably, the homologous nucleic acid of the invention hybridizes under stringent conditions to a 30 nucleic acid or longer portion

of the nucleic acid sequence of DNA164647 (SEQ ID NO:3) or its complementary sequence. preferably hybridizing under stringent conditions to a 30 nucleotide regions from nucleotide 440 to and including nucleotide 470 of SEQ ID NO:3, or its complementary sequence. In a related embodiment, the homologous nucleic acid of the invention comprises a nucleic acid sequence comprising a sequence that is at least 50%, preferably at least 80%, more preferably at least 90% homologous to the sequence from nucleotide 446 to and including nucleotide 463 of SEQ ID NO:3, or a sequence from nucleotide 2297 to and including 2337 of SEQ ID NO:3, or both sequences. Most preferably, the isolated nucleic acid comprises a sequence from nucleotide 446 to and including nucleotide 464 and/or from nucleotide 2297 to and including 2337. According to the presently disclosed invention. the isolated nucleic acid of the invention comprises a sequence having at least 65%, preferably at least 75%, more preferably at least 85%, still more preferably at least 90%, and most preferably at least 96% homologous to a sequence from approximately nucleotide 412 to and including nucleotide 477 of SEQ ID NO:3, or its complementary sequence. Preferably, the sequence encodes a histidine-rich region of an antigenic polypeptide, preferably an ECD.

In another aspect, the present invention relates to an isolated polypeptide comprising an amino acid sequence having homology to the amino acid sequence (SEQ ID NO:4), or a fragment thereof. encoded by or within DNA164647, designated herein as LIV-1-164647. Preferably the homology is at least approximately 80% homology, more preferably at least approximately 90%, still more preferably at least approximately 95%, and most preferably at least approximately 97% homology. Preferably, a LIV-1-164647 amino acid sequence of the invention is an aqueous soluble ECD homologous to amino acid 1 to approximately amino acid 327 or a fragment thereof comprising at least 10 amino acids. The region of the ECD is readily determined from a standard hydropathy plot indicating the relatively more hydrophilic region N-terminal of a hydrophobic transmembrane region. In a related embodiment, the homologous amino acid sequence of the invention comprises a sequence from amino acid 126 to and including amino acid 132 of SEQ ID NO:4 (specifically, the amino acid sequence HDHHSHH (SEQ ID NO:17)), or a sequence from amino acid 743 to and including amino acid 755 of SEQ ID NO:4 (specifically, the amino acid sequence SIFEHKIVFRINF (SEQ ID NO:18), or both sequences. The present invention further includes an isolated polypeptide comprising an amino acid sequence having at least 50%, preferably at least 80%, more preferably at least 90% homologous to SEQ ID NO:17. The present invention still further includes an isolated polypeptide comprising an amino acid sequence having at least 20%, more preferably at least 50%, still more preferably at least 80%, and most preferably at least 90% homology to SEQ ID NO:18.   In still another embodiment, the invention includes an isolated nucleic acid of SEQ ID NO:3 and an isolated polypeptide of SEQ ID NO:4. The present invention further includes an isolated polypeptide comprising an amino acid sequence having at least 65%, preferably at least 75%, more preferably at least 85%, still more preferably at least 90%, and most preferably at least 96% to a sequence from amino acid 114 to and including amino acid 135 of SEQ ID NO:4. The amino acid sequence from amino acid 114 to 135 is designated SEQ ID NO:19. A still further embodiment includes an isolated polypeptide comprising SEQ ID NO:17 and/or SEQ ID NO:18.

In still another embodiment, the invention includes an isolated polypeptide comprising an amino acid sequence wherein the sequence is at least 98% homologous to the sequence from amino acid 1 to and including amino acid 327 of SEQ ID NO:4, more preferably comprising the ECD of LIV-1-164647. Most preferably, the

sequence comprises SEQ ID NO:17. forms a portion of an extracellular domain (ECD). preferably the ECD of LIV-1-164647. The term "portion" as used herein refers to a sequence that comprises at least 7 amino acids of the ECD of LIV-1-164647 from amino acid 1 to and including amino acid 327 of SEQ ID NO:4.

In another embodiment, the present invention concerns an antibody which specifically binds a LIV-1 polypeptide. Preferably. the antibody is a monoclonal antibody. More preferably. the antibody is a human antibody or a humanized antibody. In one embodiment, the antibody reduces activity of overexpressed LIV-1 polypeptide in a cell. In another aspect. the antibody is a monoclonal antibody. which preferably has nonhuman complementarity determining region (CDR) residues and human framework region (FR) residues. The antibody may be labeled and may be immobilized on a solid support. In a further aspect. the antibody is an antibody fragment, a single-chain antibody, or an anti-idiotypic antibody. Preferably, a LIV-1-binding antibody of the invention binds specifically to a polypeptide having at least approximately 80% homology. more preferably at least approximately 90% homology. still more preferably at least approximately 95% homology. and most preferably at least approximately 97% homology to the LIV-1 ECD nucleic acid sequence. or a fragment thereof, encoded within the ECD coding region (nucleotides 1-1000) of DNA164647 (SEQ ID NO:3). More preferably. a LIV-1-binding antibody of the invention binds specifically to a polypeptide having at least 80% homology. more preferably at least approximately 90% homology, still more preferably at least approximately 95% homology. and most preferably at least approximately 97% homology to the amino acid sequence of LIV-1 ECD (amino acids 1-327 of SEQ ID NO:4). In a preferred embodiment. the present invention concerns an isolated antibody which specifically binds a LIV-1 polypeptide encoded by a nucleic acid sequence comprising a nucleic acid sequence having at least 65%, preferably at least 75%. more preferably at least 85%, still more preferably at least 90%. and most preferably at least 96% homology to a sequence from nucleotide 446 to and including nucleotide 463 of SEQ ID NO:3, or the nucleic acid sequence from 2297 to and including 2337 of SEQ ID NO:3, or both nucleic acid sequences. In another preferred embodiment. the present invention concerns an isolated antibody which specifically binds a LIV-1 polypeptide comprising the amino acid sequence having at least 65%, preferably at least 75%, more preferably at least 85%. still more preferably at least 90%, and most preferably at least 96% homology to a sequence from amino acid 126 to and including amino acid 132 of SEQ ID NO:4. or the amino acid sequence from amino acid 743 to and including amino acid 755 of SEQ ID NO:4, or both amino acid sequences.

In still another embodiment. the invention concerns an antibody, preferably a monoclonal antibody. that specifically binds the same epitope of LIV-1, preferably LIV-1-1164647, that is bound by any one of the monoclonal antibodies produced by the hybridoma cell lines deposited with the American Type Culture Collection (ATCC) as disclosed herein.

In a further embodiment, the invention includes an antibody that binds to a polypeptide comprising a sequence from amino acid 1 to and including amino acid 147 of SEQ ID NO:4. In another embodiment. the antibody binds a polypeptide comprising amino acid 148 to and including amino acid 298 of SEQ ID NO:4. Preferably. the antibodies are monoclonal antibodies. More preferably, the monoclonal antibodies are human antibodies or humanized antibodies.

In another aspect. the invention concerns a composition comprising an antibody which binds LIV-1

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polypeptide in an admixture with a pharmaceutically acceptable carrier. In one aspect, the composition comprises a therapeutically effective amount of the antibody. In another aspect, the composition comprises a further active ingredient, which may, for example, be a further antibody or a cytotoxic or chemotherapeutic agent. Preferably, the composition is sterile.

5   In a further embodiment, the invention concerns a nucleic acid encoding an anti-LIV-1 antibody according to the invention, and vectors and recombinant host cells comprising such nucleic acid.

In a still further embodiment, the invention concerns a method for producing an anti-LIV-1 antibody by culturing a host cell transformed with nucleic acid encoding the antibody under conditions such that the antibody is expressed, and recovering the antibody from the cell culture.

10   The invention further concerns antagonists and agonists of a LIV-1 polypeptide, which antagonists inhibit one or more of the functions or activities of the LIV-1 polypeptide and which agonists mimic one or more of the functions or activities of the LIV-1 polypeptide. Preferably the LIV-1 polypeptide is the LIV-1-164647 polypeptide whose functions or activities are antagonized or agonized.

In another embodiment, the invention concerns a method for determining the presence of a LIV-1

15   polypeptide or fragment thereof comprising exposing a cell suspected of containing the LIV-1 polypeptide to an anti-LIV-1 antibody of the invention and determining binding of the antibody to the cell.

In yet another embodiment, the present invention concerns a method of diagnosing tumor in a mammal, comprising detecting the level of expression of a gene encoding a LIV-1 polypeptide in a test sample of tissue cells obtained from the mammal, and in a control sample of known normal tissue cells of the same cell type, wherein a

20   higher expression level in the test sample indicates the presence of tumor in the mammal from which the test tissue cells were obtained.

In another embodiment, the present invention concerns a method of diagnosing tumor in a mammal, comprising contacting an anti-LIV-1 antibody with a test sample of tissue cells obtained from the mammal, and detecting the formation of a complex between the anti-LIV-1 antibody and the LIV-1 polypeptide in the test sample.

25   The detection may be qualitative or quantitative, and may be performed in comparison with monitoring the complex formation in a control sample of known normal tissue cells of the same cell type. A larger quantity of complexes formed in the test sample indicates the presence of tumor in the mammal from which the test tissue cells were obtained. The antibody preferably carries a detectable label. Complex formation can be monitored, for example, by light microscopy, flow cytometry, fluorimetry, or other techniques known in the art.

30   For the methods of diagnosing, the test sample is usually obtained from an individual suspected to have neoplastic cell growth or proliferation (e.g. cancerous cells).

In another embodiment, the invention involves a method of diagnosing breast tumor tissue as tissue that overexpresses LIV-1 protein and expresses normal levels of ErbB2. The method comprises providing a test sample of tissue suspected of being cancerous, contacting the test sample with an antibody to the naturally occurring form

35   of the LIV-1 gene product, contacting the same or duplicate test sample with an anti-ErbB2 antibody, detecting the relative binding of the antibodies to the test sample compared to a control sample, where the control may be a positive control, a negative control, or both. A test sample that overexpresses LIV-1 gene product (relative to normal

tissue), but does not overexpress ErbB2 protein (relative to normal tissue), is diagnosed as a member of the population of breast tumors to be treated by the compositions and methods of the invention. Useful in the diagnostic assay method of the invention are anti-ErbB2 antibodies that bind the extracellular domain of the ErbB2 receptor. and preferably bind to the epitope 4D5 or 3H4 within the ErbB2 extracellular domain sequence. More preferably, the antibody is the antibody 4D5. Other preferred ErbB2-binding antibodies include. but are not limited to. antibodies 7C2, 7F3, and 2C4. Information regarding anti-ErbB2 antibodies is found. for example. in Hudziak, R.M. et al., U.S. Patent No. 5,772,997, incorporated herein by reference in its entirety.

In another aspect, the present invention concerns a cancer diagnostic kit, comprising an anti-LIV-1-164647 antibody and a carrier (e.g. a buffer) in suitable packaging. The kit preferably contains instructions for using the antibody to detect the LIV-1 polypeptide.

In yet another aspect, the invention concerns a method for inhibiting the growth of tumor cells comprising exposing a cell which overexpresses a LIV-1 polypeptide to an effective amount of an agent inhibiting the expression and/or activity of the LIV-1 polypeptide. The agent preferably is an anti-LIV-1-164647 antibody. a small organic and inorganic molecule, peptide, phosphopeptide. antisense or ribozyme molecule. or a triple helix molecule. In a specific aspect, the agent, e.g. anti-LIV-1-164647 antibody induces cell death. or at least slows cell growth sufficiently to allow other methods of cancer treatment to be administered. In a further aspect. the tumor cells are further exposed to radiation treatment and/or a cytotoxic or chemotherapeutic agent.

In yet another aspect, the invention concerns a method for the treatment of a human patient susceptible to or diagnosed with a disorder characterized by overexpression of LIV-1 gene product without overexpression of ErbB2 receptor. In an embodiment, the method comprises administering a therapeutically effective amount of an anti-LIV-1 polypeptide antibody, where administration may be intravenous, subcutaneous. or other pharmaceutically acceptable method of antibody delivery. Preferably the antibody specifically binds the naturally occurring form of the LIV-1-164647 polypeptide, wherein the binding is preferably to the extracellular domain or a fragment thereof. Preferably, the initial dose (or doses) as well as the subsequent maintenance dose or doses are administered subcutaneously. Optionally, where the patient's tolerance of the anti-LIV-1 antibody is unknown, the initial dose is administered by intravenous infusion, followed by subcutaneous administration of the maintenance doses if the patient's tolerance for the antibody is acceptable. According to the embodiment of the invention, the initial dose or doses is followed by subsequent doses of equal or smaller amounts of antibody at intervals sufficiently close to maintain the trough serum concentration of antibody at or above an efficacious target level. Preferably, an initial dose or individual subsequent dose does not exceed 100 mg/kg, and each subsequent dose is at least 1 µg/kg. The choice of delivery method for the initial and maintenance doses is made according to the ability of the animal or human patient to tolerate introduction of the antibody into the body. Where the antibody is well-tolerated. the time of infusion may be reduced. The choice of delivery method as disclosed for this embodiment applies to all drug delivery regimens contemplated according to the invention.

In a further aspect, the invention provides a method of treating LIV-1 gene product-overexpressing cancer (lacking overexpression of ErbB2) in a human patient comprising administering to the patient effective amounts of an anti-LIV-1 antibody (which antibody preferably binds the extracellular domain of LIV-1 gene product) and a

chemotherapeutic agent. In one embodiment of the invention. the chemotherapeutic agent in a toxoid including, but not limited to, paclitaxel and doxetaxel. In another embodiment, the chemotherapeutic agent is an anthracyline derivative including. but not limited to. doxorubicin and epirubicin. In still another embodiment, the chemotherapeutic agent is not administered to the patient simultaneously with the anti-LIV-1 antibody. One or more additional chemotherapeutic agents may also be administered to the patient.

The disorder to be treated by a method of the invention preferably is a benign or malignant tumor characterized by the overexpression of the LIV-1 gene product. Preferably, the malignant cells of the tumor express approximately the same level of ErbB2 (or less) as a non-cancerous cell of the same type. For example. the disorder to be treated is a cancer, such as breast cancer, lung cancer, and prostate cancer.

Accordingly, one aspect of the invention involves compounds that bind to the LIV-1 protein and inhibit its activity. Preferably the compounds bind to the extracellular region of the LIV-1 protein and inhibit its activity. In an embodiment of the invention. the inhibiting compounds are antibodies specific to the LIV-1 gene product or fragments thereof. Preferably, the inhibiting compounds of the invention bind specifically to the extracellular region of the LIV-1 protein.

In another aspect. the invention involves compounds that block the binding of an activating ligand of LIV-1 protein. Such ligand-blocking compounds include, but are not limited to polypeptides, proteins. antibodies and the like. Preferably the ligand-blocking compounds specifically block the activity of a LIV-1 activating ligand. More preferably, the ligand-blocking compounds of the invention inhibit growth of a LIV-1-expressing cell.

In another aspect. the invention involves a method for identifying a compound capable of inhibiting the expression and/or activity of a LIV-1 polypeptide, comprising contacting a candidate compound with a LIV-1 polypeptide under conditions and for a time sufficient to allow these two components to interact. In a specific aspect. either the candidate compound or the LIV-1 polypeptide is immobilized on a solid support. In another aspect, the non-immobilized component carries a detectable label.

In yet another aspect, the invention concerns an article of manufacture, comprising a container; a composition within the container comprising an anti-LIV-1 antibody that binds the LIV-1 protein (preferably binding to the extracellular domain or a fragment thereof) or binds an activating ligand of the LIV-1 protein; and optionally a label on or associated with the container that indicates that the composition can be used for treating a condition characterized by overexpression of LIV-1 without overexpression of ErbB2. According to another embodiment of the invention, the article of manufacture further includes a package insert comprising instructions to administer the anti-LIV-1 antibody subcutaneously for at least one of the doses, preferably for all of the subsequent doses following the initial dose. most preferably for all doses.

Where the methods and compositions of the invention comprise an anti-LIV-1 antibody. which specifically and preferably binds the extracellular domain of a LIV-1 gene product, or a fragment of the extracellular domain. The compositions of the invention preferably include a humanized LIV-1 antibody. Thus. the invention further pertains to a composition comprising an antibody that specifically and preferably binds the extracellular domain of LIV-1 gene product, and pertains to the use of the antibody for treating LIV-1+/ErbB2- expressing cancer in a human, e.g., LIV-1 overexpressing cancer that does not coexpress high levels of ErbB2. Preferably the antibody is

a monoclonal antibody. *e.g.*, humanized anti-LIV-1 monoclonal antibody that binds to the extracellular domain (or a portion of the extracellular domain) of LIV-1 (hereinafter anti-LIV-1). The antibody may be an intact antibody (*e.g.*, an intact IgG₁ antibody) or an antibody fragment (*e.g.*, a Fab. F(Ab')₂, diabody, and the like). The variable light chain and variable heavy chain regions of humanized anti-LIV-1 antibody.

5   These and other advantages and features of the present invention will become apparent to those persons skilled in the art upon reading the details of the invention as more fully set forth below and in the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1C are the nucleic acid sequence (SEQ ID NO:1 (coding sequence). Fig. 1A-1 to 1A-2) and the amino acid sequence (SEQ ID NO:2. Fig. 1B) of the LIV-1 protein. The dashed overlined portion is predicted to

10   be a signal sequence (approximately amino acids 1 to 20). The predicted extracellular domain of LIV-1 protein is that portion of the LIV-1 amino acid sequence underscored by a dashed line (approximately amino acids 24 to 312). The predicted transmembrane domain extends from approximately amino acid 318 to approximately amino acid 367. The approximate positions of the domains were predicted using a standard hydropathy analysis program. A nucleic acid sequence (SEQ ID NO:5) encoding a portion of LIV-1 and useful in microarray analysis is shown in

15   Fig. 1C.

Figs. 2A-2B are the nucleic acid sequence and amino acid sequence, respectively, corresponding to DNA164647. Fig. 2A1-2A5 is the nucleotide sequence of DNA164647 that is a cDNA encoding a native sequence LIV-1 protein. SEQ ID NO:3 is the coding strand of DNA164647. Fig. 2B is the derived amino acid sequence (SEQ ID NO:4) of a native LIV-1 polypeptide encoded by DNA164647.

20   Fig. 3-1 - 3-11 is an alignment of SEQ ID NO:1 and SEQ ID NO:3 nucleic acid sequences.

Fig. 4-1 - 4-3 is an alignment of SEQ ID NO:2 and SEQ ID NO:4 amino acid sequences. The sequences differ in the ECD (near amino acid 130 of SEQ ID NO:4) and C-terminal region beyond about amino acid 740 of SEQ ID NO:4. A single amino acid difference was found at amino acid 651 of SEQ ID NO:4.

Fig. 5 is a flow chart illustrating the FLIP cloning method up to the restriction digestion selection step, as

25   described herein. The shaded boxes flanking the vector sequence represent the target gene sequences.

Fig. 6 is a graphical representation of a fluorescent activated cell sorting (FACS) analysis in which an anti-LIV-1-164647 monoclonal antibody was shown to bind primarily to the surface of 3T3 cells transfected with DNA 164647. The term "pRK5" refers cells transfected with vector lacking a LIV-1-164647 insert. The term "pRK5-LIV-1-164647" refers to cells transfected with vector expressing full length LIV-1-164647.

30   Fig. 7 is a bar graph demonstrating that the LIV-1-164647 extracellular domain is expressed on the surface of cells transfected with DNA encoding the full-length LIV-1-164647 protein.

DESCRIPTION OF THE EMBODIMENTS

I.   Definitions

As used herein, the term "LIV-1" refers to a gene or its encoded protein. which gene transcript is detected

35   in above normal levels in some cancer cells. More specifically, a LIV-1 gene or protein of the present invention is one which is encoded by DNA164647 (SEQ ID NO:3) and has the deduced amino acid sequence of SEQ ID NO:4. As used herein, the term LIV-1 refers to LIV-1-164647 where the disclosure refers to a nucleic acid comprising

at least 21 nucleotides of SEQ ID NO:3. or where the disclosure refers to an amino acid sequence comprising at least 7 amino acids of SEQ ID NO:4 as disclosed herein. According to the invention. LIV-1-164647 is expressed in higher than normal amounts in a cell. while the gene encoding ErbB2 receptor is not expressed in higher than normal amounts. Such higher than normal expression is termed "overexpression" of a gene or protein. The term

5 "LIV-1" or "LIV-1-164647" may be used to refer to a LIV-1 gene or its encoded protein. Generally. where a protein or peptide is contemplated, the term "LIV-1 protein" will be used.

The term "LIV-1 gene product" or "LIV-1 protein" refers to the expressed protein product of the gene. preferably a polypeptide or protein form of the gene product. According to the invention. a polypeptide or protein form of the LIV-1 gene product includes a soluble form of the gene product (i.e. the extracellular domain (ECD)

10 of the LIV-1 gene product), which soluble form is useful as an antigen for raising anti-LIV-1 gene product antibodies that bind the extracellular domain of full length LIV-1 gene product and inhibit its activation. It is understood that LIV-1 gene product may also refer to the messenger RNA (mRNA) gene product and. where appropriate. the distinction between protein and mRNA is made. A LIV-1 protein according to the invention is encoded by a nucleic acid of the invention comprising a sequence at least 80% homologous to the DNA164647

15 (SEQ ID NO:3 or its complement; Fig. 2A). preferably at least approximately 90% homologous, more preferably at least approximately 95%, and most preferably at least approximately 97% homologous to SEQ ID NO:3 or its complement). or a portion thereof. A LIV-1 nucleic acid of the invention hybridizes under stringent conditions to SEQ ID NO:3 or its complement) or a portion thereof. A LIV-1 protein of the invention is at least 80% homologous to the amino acid sequence encoded by DNA164647 (LIV-1 polypeptide SEQ ID NO:4; Fig. 2B), preferably at least

20 approximately 90% homologous, more preferably at least approximately 95%, and most preferably at least approximately 97% homologous to SEQ ID NO:4. or a fragment thereof.

The terms "anti-LIV-1 antibody." "LIV-1 antibody," and grammatically analogous terms refer to an antibody which binds specifically to at least a portion of the extracellular domain of the LIV-1-164647 protein having a predicted amino acid sequence of SEQ ID NO:4 (the predicted full length amino acid sequence of LIV-1-

25 164647 gene). Preferably, the antibody binds to the extracellular domain of LIV-1 gene product, more preferably binding to the same epitope as epitopes A. B. or C to which the monoclonal antibodies disclosed herein bind. Even more preferably. the anti-LIV-1-164647 antibody binds to a polypeptide having at least 65% homology to a sequence from amino acid 114 to and including amino acid 135 of SEQ ID NO:4. Preferably. an anti-LIV-1 antibody of the invention is human or humanized when the antibody is to be used to treat a human patient.

30 The antibody of the invention is preferably one which binds specifically to human LIV-1-164647, meaning that it does not significantly cross-react with other proteins. In such embodiments. the extent of binding of the antibody to proteins other than LIV-1 gene product will be less than about 10% as determined by fluorescence activated cell sorting (FACS) analysis or radioimmunoprecipitation (RIA).

The term "antibody" is used in the broadest sense and specifically covers. for example, single anti-LIV-1

35 monoclonal antibodies (including agonist. antagonist. and neutralizing antibodies). anti-LIV-1 antibody compositions with polyepitopic specificity, single chain anti-LIV-1 antibodies. The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies. i.e.. the

individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts.

"Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas. The term "antibody" is used in the broadest sense and specifically covers, without limitation, intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g.. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Native antibodies" and "native immunoglobulins" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a -sheet configuration, connected by three CDRs, which form loops connecting. and in some cases forming part of. the -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., NIH Publ. No.91-3242, Vol. I, pages 647-669 (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" to "CDR" (i.e., residues in the light chain variable domain and residues in the heavy chain variable domain; Kabat et al., Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institute of Health, Bethesda, MD. [1991]) and/or those residues from a "hypervariable loop" (i.e., residues in the light chain variable domain and residues in the heavy chain variable domain : Clothia and

Lesk, J. Mol. Biol., 196:901-917 [1987]). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')₂, and Fv fragments;
5   diabodies; linear antibodies (Zapata et al., Protein Eng., 8(10):1057-1062 [1995]); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')₂ fragment that has two antigen-combining sites and is still capable of
10   cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and -binding site. This region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody.
15   However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab fragments differ from Fab' fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH
20   is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')₂ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant
25   domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called α, δ, ε,
30   γ, and μ, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are
35   highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their

specificity. the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies. and is not to be construed as requiring production of the antibody by any particular method. For example. the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al..* Nature, 256:495 [1975], or may be made by recombinant DNA methods (see, *e.g..* U.S. Patent No. 4,816,567). The monoclonal antibodies may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.,* Nature, 352:624-628 [1991] and Marks *et al.,* J. Mol. Biol., 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass. as well as fragments of such antibodies. so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al..* Proc. Natl. Acad. Sci. USA, 81:6851-6855 [1984]). Chimeric antibodies of interest herein include human constant region sequences together with antigen binding regions of rodent (e.g. murine) origin. or "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g. Old World Monkey. Ape. macaque. etc.). or antigen binding regions derived from antibodies generated in other non-human species that have been immunized with the antigen of interest.

"Humanized" forms of non-human (*e.g.,* rodent) antibodies are chimeric immunoglobulins. immunoglobulin chains or fragments thereof (such as Fv. Fab. Fab'. F(ab')$_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part. humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity. affinity. and capacity. In some instances. framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore. humanized antibodies may comprise residues which are found neither in the recipient antibody nor in the donor antibody. These modifications are made to further refine and maximize antibody performance. In general. the humanized antibody will comprise substantially all of at least one. and typically two, variable domains. in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc). typically that of a human immunoglobulin. For further details. see, Jones *et al.,* Nature, 321:522-525 (1986); Reichmann *et al.,* Nature, 332:323-329 [1988]; and Presta, *Curr. Op. Struct. Biol.,* 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V$_H$ and V$_L$ domains of antibody. wherein these domains are present in a single polypeptide chain. Preferably. the Fv polypeptide further comprises a

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polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, Proc. Natl. Acad. Sci. USA, 90:6444-6448 (1993).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antibody includes the antibody *in situ* within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The term "overexpression," as used herein refers to overexpression of a gene and/or its encoded protein in a cell, such as a cancer cell. A cancer cell that "overexpresses" a protein is one that has significantly higher levels of that protein compared to a noncancerous cell of the same tissue type. For example, according to the present invention, the overexpression of a protein LIV-1 protein may be caused by gene amplification or by increased transcription or translation.

Overexpression of a LIV-1 protein may be determined in a diagnostic or prognostic assay by evaluating increased levels of a LIV-1 mRNA in a cell or tissue (*e.g.* via a quantitative PCR method) or by detecting a LIV-1 protein present on the surface of a cell (*e.g.* via an immunohistochemistry assay). Alternatively, or additionally, one may measure levels of LIV-1-encoding nucleic acid in the cell, *e.g.* via fluorescent *in situ* hybridization (FISH; see WO98/45479 published October, 1998), southern blotting, or polymerase chain reaction (PCR) techniques, such as real time quantitative PCR (RT-PCR). One may also study LIV-1 overexpression by measuring shed antigen (*e.g.*, LIV-1 extracellular domain) in a biological fluid such as serum by contacting the fluid or other sample with an antibody that binds to a LIV-1 protein or fragment thereof. Various *in vitro* and *in vivo* assays are available to the skilled practitioner. For example, one may expose a fluid or tissue comprising a LIV-1 protein or fragment thereof, or cells within the body of the patient to an antibody which is optionally labeled with a detectable label, *e.g.* a radioactive isotope, and binding of the antibody to cells in a sample or the patient can be evaluated for

overexpression. *e.g.* by external scanning for radioactivity or by analyzing a biopsy taken from a patient previously exposed to the antibody.

A cell that "overexpresses" LIV-1 has significantly higher than normal LIV-1 nucleic acid levels compared to a noncancerous cell of the same tissue type. Typically, the cell is a cancer cell. *e.g.* a breast, ovarian, prostate,
5   stomach, endometrial, salivary gland, lung, kidney, colon, thyroid, pancreatic or bladder cell. The cell may also be a cell line such as SKBR3, BT474, Calu 3, MDA-MB-453, MDA-MB-361 or SKOV3.

Conversely, a cancer that is "not characterized by overexpression of a LIV-1 protein or a LIV-1 gene is one which, in a diagnostic assay, does not express higher than normal levels of LIV-1 gene or LIV-1 protein compared to a noncancerous cell of the same tissue type.

10   The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include breast cancer, prostate cancer, colon cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer,
15   hepatoma, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

The phrases "gene amplification" and "gene duplication" are used interchangeably and refer to a process by which multiple copies of a gene or gene fragment are formed in a particular cell or cell line. The duplicated region (a stretch of amplified DNA) is often referred to as "amplicon." Usually, the amount of the messenger RNA
20   (mRNA) produced, *i.e.*, the level of gene expression, also increases in the proportion of the number of copies made of the particular gene expressed.

"Tumor", as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

"Treatment" is an intervention performed with the intention of preventing the development or altering the
25   pathology of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. In tumor (*e.g.*, cancer) treatment, a therapeutic agent may directly decrease the pathology of tumor cells, or render the tumor cells more susceptible to treatment by other therapeutic agents, *e.g.*, radiation and/or chemotherapy.

30   The "pathology" of cancer includes all phenomena that compromise the well-being of the patient. This includes, without limitation, abnormal or uncontrollable cell growth, metastasis, interference with the normal functioning of neighboring cells, release of cytokines or other secretory products at abnormal levels, suppression or aggravation of inflammatory or immunological response, etc.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans,
35   domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cattle, pigs, sheep, etc. Preferably, the mammal is human.

"Carriers" as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are

nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin. gelatin, or

5   immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine. glutamine. asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose. mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, polyethylene glycol (PEG), and PLURONICS™.

10   Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g., I^{131}, I^{125}, Y^{90} and Re^{186}), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial. fungal, plant or animal

15   origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include adriamycin, doxorubicin, epirubicin. 5-fluorouracil, cytosine arabinoside ("Ara-C"), cyclophosphamide, thiotepa, busulfan, cytoxin, taxoids, e.g., paclitaxel (Taxol, Bristol-Myers Squibb Oncology, Princeton, NJ), and doxetaxel (Taxotere, Rhône-Poulenc Rorer, Antony, Rnace), toxotere, methotrexate,

20   cisplatin, melphalan, vinblastine, bleomycin, etoposide, ifosfamide, mitomycin C, mitoxantrone. vincristine, vinorelbine, carboplatin, teniposide, daunomycin, carminomycin, aminopterin, dactinomycin, mitomycins, esperamicins (see U.S. Pat. No. 4,675,187), 5-FU, 6-thioguanine, 6-mercaptopurine, actinomycin D. VP-16, chlorambucil, melphalan, and other related nitrogen mustards. Also included in this definition are hormonal agents that act to regulate or inhibit hormone action on tumors such as tamoxifen and onapristone.

25   A "growth inhibitory agent" when used herein refers to a compound or composition which inhibits growth of a cell, especially cancer cell overexpressing any of the genes identified herein, either in vitro or in vivo. Thus, the growth inhibitory agent is one which significantly reduces the percentage of cells overexpressing such genes in S phase. Examples of growth inhibitory agents include agents that block cell cycle progression (at a place other than S phase), such as agents that induce G1 arrest and M-phase arrest. Classical M-phase blockers include the

30   vincas (vincristine and vinblastine), taxol, and topo II inhibitors such as doxorubicin, epirubicin, daunorubicin, etoposide, and bleomycin. Those agents that arrest G1 also spill over into S-phase arrest, for example, DNA alkylating agents such as tamoxifen, prednisone, dacarbazine, mechlorethamine, cisplatin, methotrexate, 5-fluorouracil, and ara-C. Further information can be found in The Molecular Basis of Cancer, Mendelsohn and Israel, eds., Chapter 1, entitled "Cell cycle regulation, oncogens, and antineoplastic drugs" by Murakami et al., (WB

35   Saunders: Philadelphia, 1995), especially p. 13.

"Doxorubicin" is an athracycline antibiotic. The full chemical name of doxorubicin is (8S-cis)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxo-hexopyranosyl)oxy]-7,8,9,10-tetrahydro-6,8,11-trihydroxy-8-(hydroxyacetyl)-1-

methoxy-5.12-naphthacenedione.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human

5 growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-β;

10 platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α. -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used

15 herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or converted into the more active parent form. See, e.g., Wilman, "Prodrugs in

20 Cancer Chemotherapy" *Biochemical Society Transactions.* 14. pp. 375-382. 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery." *Directed Drug Delivery.* Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include. but are not limited to. phosphate-containing prodrugs, thiophosphate-containing prodrugs. sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs, β-lactam-containing prodrugs. optionally

25 substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs. 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include. but are not limited to. those chemotherapeutic agents described above.

An "effective amount" of a polypeptide disclosed herein or an antagonist thereof. in reference to inhibition

30 of neoplastic cell growth, tumor growth or cancer cell growth. is an amount capable of inhibiting, to some extent. the growth of target cells. The term includes an amount capable of invoking a growth inhibitory, cytostatic and/or cytotoxic effect and/or apoptosis of the target cells. An "effective amount" of a LIV-1 polypeptide antagonist for purposes of inhibiting neoplastic cell growth, tumor growth or cancer cell growth. may be determined empirically and in a routine manner.

35 A "therapeutically effective amount", in reference to the treatment of tumor. refers to an amount capable of invoking one or more of the following effects: (1) inhibition. to some extent. of tumor growth, including. slowing down and complete growth arrest; (2) reduction in the number of tumor cells; (3) reduction in tumor size; (4)

inhibition (i.e.. reduction. slowing down or complete stopping) of tumor cell infiltration into peripheral organs: (5) inhibition (i.e.. reduction. slowing down or complete stopping) of metastasis: (6) enhancement of anti-tumor immune response. which may. but does not have to. result in the regression or rejection of the tumor; and/or (7) relief. to some extent. of one or more symptoms associated with the disorder. A "therapeutically effective amount" of a LIV-I polypeptide antagonist for purposes of treatment of tumor may be determined empirically and in a routine manner.

A "growth inhibitory amount" of a LIV-I antagonist is an amount capable of inhibiting the growth of a cell. especially tumor, e.g., cancer cell, either in vitro or in vivo. A "growth inhibitory amount" of a LIV-I antagonist for purposes of inhibiting neoplastic cell growth may be determined empirically and in a routine manner.

A "cytotoxic amount" of a LIV-I antagonist is an amount capable of causing the destruction of a cell. especially tumor, e.g., cancer cell. either in vitro or in vivo. A "cytotoxic amount" of a LIV-I antagonist for purposes of inhibiting neoplastic cell growth may be determined empirically and in a routine manner.

"Percent (%) amino acid sequence homology or identity" with respect to the LIV-I polypeptide sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in a LIV-I sequence. after aligning the sequences and introducing gaps. if necessary. to achieve the maximum percent sequence identity. and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art. for instance, using publicly available computer software such as BLAST. BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. For purposes herein, however. % amino acid sequence identity values are obtained as described below by using the sequence comparison computer program ALIGN-2. wherein the complete source code for the ALIGN-2 program was authored by Genentech. Inc.. and the source code shown in Figures 20A-Q has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. and is provided in Table I. The ALIGN-2 program is publicly available through Genentech, Inc.. South San Francisco. California. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

For purposes herein. the % amino acid sequence identity of a given amino acid sequence A to, with. or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B. and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid

sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

Unless specifically stated otherwise, all % amino acid sequence homology or identity values used herein are obtained as described above using the ALIGN-2 sequence comparison computer program. However, % amino acid sequence identity may also be determined using the sequence comparison program NCBI-BLAST2 (Altschul et al., Nucleic Acids Res., 25:3389-3402 (1997)). The NCBI-BLAST2 sequence comparison program may be downloaded from http://www.ncbi.nlm.nih.gov. NCBI-BLAST2 uses several search parameters, wherein all of those search parameters are set to default values including, for example, unmask = yes, strand = all, expected occurrences = 10, minimum low complexity length = 15/5, multi-pass e-value = 0.01, constant for multi-pass = 25, dropoff for final gapped alignment = 25 and scoring matrix = BLOSUM62.

In situations where NCBI-BLAST2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program NCBI-BLAST2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

In addition, % amino acid sequence identity may also be determined using the WU-BLAST-2 computer program (Altschul et al., Methods in Enzymology, 266:460-480 (1996)). Most of the WU-BLAST-2 search parameters are set to the default values. Those not set to default values, i.e., the adjustable parameters, are set with the following values: overlap span = 1, overlap fraction = 0.125, word threshold (T) = 11, and scoring matrix = BLOSUM62. For purposes herein, a % amino acid sequence identity value is determined by dividing (a) the number of matching identical amino acids residues between the amino acid sequence of the LIV-1 polypeptide of interest having a sequence derived from the native LIV-1 polypeptide encoded by DNA164647 and the comparison amino acid sequence of interest (i.e., the sequence against which the LIV-1 polypeptide of interest is being compared which may be a LIV-1 variant polypeptide) as determined by WU-BLAST-2 by (b) the total number of amino acid residues of the LIV-1 polypeptide of interest. For example, in the statement "a polypeptide comprising an amino acid sequence A which has or having at least 80% amino acid sequence identity to the amino acid sequence B", the amino acid sequence A is the comparison amino acid sequence of interest and the amino acid sequence B is the amino acid sequence of the LIV-1 polypeptide of interest.

"Percent (%) nucleic acid sequence homology or identity" with respect to the LIV-1 polypeptide-encoding nucleic acid sequences identified herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a LIV-1 polypeptide-encoding nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of

determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN, ALIGN-2 or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. For purposes herein, however, % nucleic acid sequence identity values are obtained as described below by using the sequence comparison computer program ALIGN-2, wherein the complete source code for the ALIGN-2 program is has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087, and is provided herein in Table 1 as source code. The ALIGN-2 program is publicly available through Genentech, Inc., South San Francisco, California. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

<u>Table 1</u>

```
/*
 *
 * C-C increased from 12 to 15
 * Z is average of EQ
 * B is average of ND
 * match with stop is _M: stop-stop = 0; J (joker) match = 0
 */
#define _M      -8          /* value of a match with a stop */

int     _day[26][26] = {
/*      A B C D E F G H I J K L M N O P Q R S T U V W X Y Z */
/* A */ { 2, 0,-2, 0, 0,-4, 1,-1,-1, 0,-1,-2,-1, 0,_M, 1, 0,-2, 1, 1, 0, 0,-6, 0,-3, 0},
/* B */ { 0, 3,-4, 3, 2,-5, 0, 1,-2, 0, 0,-3,-2, 2,_M,-1, 1, 0, 0, 0, 0,-2,-5, 0,-3, 1},
/* C */ {-2,-4,15,-5,-5,-4,-3,-3,-2, 0,-5,-6,-5,-4,_M,-3,-5,-4, 0,-2, 0,-2,-8, 0, 0,-5},
/* D */ { 0, 3,-5, 4, 3,-6, 1, 1,-2, 0, 0,-4,-3, 2,_M,-1, 2,-1, 0, 0, 0,-2,-7, 0,-4, 2},
/* E */ { 0, 2,-5, 3, 4,-5, 0, 1,-2, 0, 0,-3,-2, 1,_M,-1, 2,-1, 0, 0, 0,-2,-7, 0,-4, 3},
/* F */ {-4,-5,-4,-6,-5, 9,-5,-2, 1, 0,-5, 2, 0,-4,_M,-5,-5,-4,-3,-3, 0,-1, 0, 0, 7,-5},
/* G */ { 1, 0,-3, 1, 0,-5, 5,-2,-3, 0,-2,-4,-3, 0,_M,-1,-1,-3, 1, 0, 0,-1,-7, 0,-5, 0},
/* H */ {-1, 1,-3, 1, 1,-2, 2, 6,-2, 0, 0,-2,-2, 2,_M, 0, 3, 2,-1,-1, 0,-2,-3, 0, 0, 2},
/* I */ {-1,-2,-2,-2,-2, 1,-3,-2, 5, 0,-2, 2, 2,-2,_M,-2,-2,-2,-1, 0, 0, 4,-5, 0,-1,-2},
/* J */ { 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,_M, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0},
/* K */ {-1, 0,-5, 0, 0,-5,-2, 0,-2, 0, 5,-3, 0, 1,_M,-1, 1, 3, 0, 0, 0,-2,-3, 0,-4, 0},
/* L */ {-2,-3,-6,-4,-3, 2,-4,-2, 2, 0,-3, 6, 4,-3,_M,-3,-2,-3,-3,-1, 0, 2,-2, 0,-1,-2},
/* M */ {-1,-2,-5,-3,-2, 0,-3,-2, 2, 0, 0, 4, 6,-2,_M,-2,-1, 0,-2,-1, 0, 2,-4, 0,-2,-1},
/* N */ { 0, 2,-4, 2, 1,-4, 0, 2,-2, 0, 1,-3,-2, 2,_M,-1, 1, 0, 1, 0, 0,-2,-4, 0,-2, 1},
/* O */ {_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M, 0,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M,_M},
/* P */ { 1,-1,-3,-1,-1,-5,-1, 0,-2, 0,-1,-3,-2,-1,_M, 6, 0, 0, 1, 0, 0,-1,-6, 0,-5, 0},
/* Q */ { 0, 1,-5, 2, 2,-5,-1, 3,-2, 0, 1,-2,-1, 1,_M, 0, 4, 1,-1,-1, 0,-2,-5, 0,-4, 3},
/* R */ {-2, 0,-4,-1,-1,-4,-3,-2, 0, 0, 3,-3, 0, 0,_M, 0, 1, 6, 0,-1, 0,-2, 2, 0,-4, 0},
/* S */ { 1, 0, 0, 0, 0,-3, 1,-1,-1, 0, 0,-3,-2, 1,_M, 1,-1, 0, 2, 1, 0,-1,-2, 0,-3, 0},
/* T */ { 1, 0,-2, 0, 0,-3, 0,-1, 0, 0, 0,-1,-1, 0,_M, 0,-1,-1, 1, 3, 0, 0,-5, 0,-3, 0},
/* U */ { 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,_M, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0},
/* V */ { 0,-2,-2,-2,-2,-1,-1,-2, 4, 0,-2, 2, 2,-2,_M,-1,-2,-2,-1, 0, 0, 4,-6, 0,-2,-2},
/* W */ {-6,-5,-8,-7,-7, 0,-7,-3,-5, 0,-3,-2,-4,-4,_M,-6,-5, 2,-2,-5, 0,-6,17, 0, 0,-6},
/* X */ { 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0,_M, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0},
/* Y */ {-3,-3, 0,-4,-4, 7,-5, 0,-1, 0,-4,-1,-2,-2,_M,-5,-4,-4,-3,-3, 0,-2, 0, 0,10,-4},
/* Z */ { 0, 1,-5, 2, 3,-5, 0, 2,-2, 0, 0,-2,-1, 1,_M, 0, 3, 0, 0, 0, 0,-2,-6, 0,-4, 4}
};
```

Page 1 of day.h

```
/*
 */
#include <stdio.h>
#include <ctype.h>

#define MAXJMP   16      /* max jumps in a diag */
#define MAXGAP   24      /* don't continue to penalize gaps larger than this */
#define JMPS     1024    /* max jmps in an path */
#define MX       4       /* save if there's at least MX-1 bases since last jmp */

#define DMAT     3       /* value of matching bases */
#define DMIS     0       /* penalty for mismatched bases */
#define DINS0    8       /* penalty for a gap */
#define DINS1    1       /* penalty per base */
#define PINS0    8       /* penalty for a gap */
#define PINS1    4       /* penalty per residue */
```

20

```
struct jmp {
        short           n[MAXJMP]:      /* size of jmp (neg for dely) */
        unsigned short  x[MAXJMP]:      /* base no. of jmp in seq x */
};                                      /* limits seq to 2^16 -1 */

struct diag {
        int             score:          /* score at last jmp */
        long            offset;         /* offset of prev block */
        short           ijmp:           /* current jmp index */
        struct jmp      jp:             /* list of jmps */
};

struct path {
        int     spc:                    /* number of leading spaces */
        short   n[JMPS]:/* size of jmp (gap) */
        int     x[JMPS]:/* loc of jmp (last elem before gap) */
};

char            *ofile;                 /* output file name */
char            *namex[2]:              /* seq names: getseqs() */
char            *prog;                  /* prog name for err msgs */
char            *seqx[2]:               /* seqs: getseqs() */
int             dmax;                   /* best diag: nw() */
int             dmax0;                  /* final diag */
int             dna;                    /* set if dna: main() */
int             endgaps;                /* set if penalizing end gaps */
int             gapx, gapy;             /* total gaps in seqs */
int             len0, len1:             /* seq lens */
int             ngapx, ngapy;           /* total size of gaps */
int             smax;                   /* max score: nw() */
int             *xbm;                   /* bitmap for matching */
long            offset;                 /* current offset in jmp file */
struct  diag    *dx;                    /* holds diagonals */
struct  path    pp[2];                  /* holds path for seqs */

char            *calloc(), *malloc(), *index(), *strcpy();
char            *getseq(), *g_calloc():
```

Page 1 of nw.h

```
/* Needleman-Wunsch alignment program
 *
 * usage: progs file1 file2
 *    where file1 and file2 are two dna or two protein sequences.
 *    The sequences can be in upper- or lower-case an may contain ambiguity
 *    Any lines beginning with ';'. '>' or '<' are ignored
 *    Max file length is 65535 (limited by unsigned short x in the jmp struct)
 *    A sequence with 1/3 or more of its elements ACGTU is assumed to be DNA
 *    Output is in the file "align.out"
 *
 * The program may create a tmp file in /tmp to hold info about traceback.
 * Original version developed under BSD 4.3 on a vax 8650
 */
#include "nw.h"
#include "day.h"
```

```
static    _dbval[26] = {
          1,14,2,13,0,0,4,11,0,0,12,0,3,15,0,0,0,5,6,8,8,7,9,0,10,0
};


static    _pbval[26] = {
          1, 2|(1<<('D'-'A'))|(1<<('N'-'A')), 4, 8, 16, 32, 64,
          128, 256, 0xFFFFFFFF, 1<<10, 1<<11, 1<<12, 1<<13, 1<<14,
          1<<15, 1<<16, 1<<17, 1<<18, 1<<19, 1<<20, 1<<21, 1<<22,
          1<<23, 1<<24, 1<<25|(1<<('E'-'A'))|(1<<('Q'-'A'))
};


main(ac, av)                                                                main
          int       ac;
          char      *av[];
{
          prog = av[0];
          if (ac != 3) {
                    fprintf(stderr,"usage: %s file1 file2\n", prog);
                    fprintf(stderr,"where file1 and file2 are two dna or two protein sequences.\n");
                    fprintf(stderr,"The sequences can be in upper- or lower-case\n");
                    fprintf(stderr,"Any lines beginning with ';' or '<' are ignored\n");
                    fprintf(stderr,"Output is in the file \"align.out\"\n");
                    exit(1);
          }
          namex[0] = av[1];
          namex[1] = av[2];
          seqx[0] = getseq(namex[0], &len0);
          seqx[1] = getseq(namex[1], &len1);
          xbm = (dna)? _dbval : _pbval;

          endgaps = 0;                      /* ] to penalize endgaps */
          ofile = "align.out";              /* output file */

          nw();                   /* fill in the matrix, get the possible jmps */
          readjmps();             /* get the actual jmps */
          print();                /* print stats, alignment */

          cleanup(0);             /* unlink any tmp files */
}
```

                                                                Page 1 of nw.c

```
/* do the alignment, return best score: main()
 * dna: values in Fitch and Smith, PNAS, 80, 1382-1386, 1983
 * pro: PAM 250 values
 * When scores are equal, we prefer mismatches to any gap, prefer
 * a new gap to extending an ongoing gap, and prefer a gap in seqx
 * to a gap in seq y.
 */
nw()                                                                        nw
{
          char      *px, *py;          /* seqs and ptrs */
          int       *ndely, *dely;     /* keep track of dely */
          int       ndelx, delx;       /* keep track of delx */
          int       *tmp;              /* for swapping row0, row1 */
          int       mis;               /* score for each type */
          int       ins0, ins1;        /* insertion penalties */
```

```
        register       id:             /* diagonal index */
        register       ij:             /* jmp index */
        register       *col0, *col1:    /* score for curr. last row */
        register       xx, yy;         /* index into seqs */

        dx = (struct diag *)g_calloc("to get diags", len0+len1+1, sizeof(struct diag));

        ndely = (int *)g_calloc("to get ndely", len1+1, sizeof(int));
        dely = (int *)g_calloc("to get dely", len1+1, sizeof(int));
        col0 = (int *)g_calloc("to get col0", len1+1, sizeof(int));
        col1 = (int *)g_calloc("to get col1", len1+1, sizeof(int));
        ins0 = (dna)? DINS0 : PINS0;
        ins1 = (dna)? DINS1 : PINS1;

        smax = -10000;
        if (endgaps) {
                for (col0[0] = dely[0] = -ins0, yy = 1; yy <= len1; yy++) {
                        col0[yy] = dely[yy] = col0[yy-1] - ins1;
                        ndely[yy] = yy;
                }
                col0[0] = 0;            /* Waterman Bull Math Biol 84 */
        }
        else
                for (yy = 1; yy <= len1; yy++)
                        dely[yy] = -ins0;

        /* fill in match matrix
         */
        for (px = seqx[0], xx = 1; xx <= len0; px++, xx++) {
                /* initialize first entry in col
                 */
                if (endgaps) {
                        if (xx == 1)
                                col1[0] = delx = -(ins0+ins1);
                        else
                                col1[0] = delx = col0[0] - ins1;
                        ndelx = xx;
                }
                else {
                        col1[0] = 0;
                        delx = -ins0;
                        ndelx = 0;
                }
```

Page 2 of nw.c

...nw

```
        for (py = seqx[1], yy = 1; yy <= len1; py++, yy++) {
                mis = col0[yy-1];
                if (dna)
                        mis += (xbm[*px-'A']&xbm[*py-'A'])? DMAT : DMIS;
                else
                        mis += _day[*px-'A'][*py-'A'];

                /* update penalty for del in x seq;
                 * favor new del over ongong del
                 * ignore MAXGAP if weighting endgaps
                 */
                if (endgaps || ndely[yy] < MAXGAP) {
```

```
                                    if (col0[yy] - ins0 >= dely[yy]) {
                                            dely[yy] = col0[yy] - (ins0+ins1):
                                            ndely[yy] = 1:
                                    } else {
                                            dely[yy] -= ins1:
                                            ndely[yy]++:
                                    }
                            } else {
                                    if (col0[yy] - (ins0+ins1) >= dely[yy]) {
                                            dely[yy] = col0[yy] - (ins0+ins1):
                                            ndely[yy] = 1:
                                    } else
                                            ndely[yy]++:
                            }


                            /* update penalty for del in y seq:
                             * favor new del over ongong del
                             */
                            if (endgaps || ndelx < MAXGAP) {
                                    if (col1[yy-1] - ins0 >= delx) {
                                            delx = col1[yy-1] - (ins0+ins1):
                                            ndelx = 1:
                                    } else {
                                            delx -= ins1:
                                            ndelx++:
                                    }
                            } else {
                                    if (col1[yy-1] - (ins0+ins1) >= delx) {
                                            delx = col1[yy-1] - (ins0+ins1):
                                            ndelx = 1:
                                    } else
                                            ndelx++:
                            }


                            /* pick the maximum score: we're favoring
                             * mis over any del and delx over dely
                             */
```

```
                    id = xx - yy + len1 - 1:
                    if (mis >= delx && mis >= dely[yy])
                            col1[yy] = mis:
                    else if (delx >= dely[yy]) {
                            col1[yy] = delx;
                            ij = dx[id].ijmp;
                            if (dx[id].jp.n[0] && (!dna || (ndelx >= MAXJMP
                            && xx > dx[id].jp.x[ij]+MX) || mis > dx[id].score+DINS0)) {
                                    dx[id].ijmp++;
                                    if (++ij >= MAXJMP) {
                                            writejmps(id);
                                            ij = dx[id].ijmp = 0:
                                            dx[id].offset = offset:
                                            offset += sizeof(struct jmp) + sizeof(offset):
                                    }
                            }
                            dx[id].jp.n[ij] = ndelx:
                            dx[id].jp.x[ij] = xx:
                            dx[id].score = delx;
                    }
```

```
                        else {
                                coll[yy] = dely[yy];
                                ij = dx[id].ijmp;

5           if (dx[id].jp.n[0] && (!dna || (ndely[yy] >= MAXJMP
                                && xx > dx[id].jp.x[ij]+MX) || mis > dx[id].score+DINS0)) {
                                        dx[id].ijmp++;
                                        if (++ij >= MAXJMP) {
                                                writejmps(id);
10                                              ij = dx[id].ijmp = 0;
                                                dx[id].offset = offset;
                                                offset += sizeof(struct jmp) + sizeof(offset);
                                        }
                                }
15                              dx[id].jp.n[ij] = -ndely[yy];
                                dx[id].jp.x[ij] = xx;
                                dx[id].score = dely[yy];
                        }
                        if (xx == len0 && yy < len1) {
20                              /* last col
                                */
                                if (endgaps)
                                        coll[yy] -= ins0+ins1*(len1-yy);
                                if (coll[yy] > smax) {
25                                      smax = coll[yy];
                                        dmax = id;
                                }
                        }
                }
30              if (endgaps && xx < len0)
                        coll[yy-1] -= ins0+ins1*(len0-xx);
                if (coll[yy-1] > smax) {
                        smax = coll[yy-1];
                        dmax = id;
35              }
                tmp = col0; col0 = coll; coll = tmp;
        }
        (void) free((char *)ndely);
        (void) free((char *)dely);
40      (void) free((char *)col0);
        (void) free((char *)coll);
}
```

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```
45      /*
        *
        * print() -- only routine visible outside this module
        *
        * static:
50      * getmat() -- trace back best path, count matches: print()
        * pr_align() -- print alignment of described in array p[]: print()
        * dumpblock() -- dump a block of lines with numbers, stars: pr_align()
        * nums() -- put out a number line: dumpblock()
        * putline() -- put out a line (name, [num], seq, [num]): dumpblock()
55      * stars() - -put a line of stars: dumpblock()
        * stripname() -- strip any path and prefix from a seqname
        */

        #include "nw.h"
60
```

```
        #define SPC        3
        #define P_LINE     256      /* maximum output line */
        #define P_SPC      3        /* space between name or num and seq */


5       extern  _day[26][26];
        int     olen;              /* set output line length */
        FILE    *fx;               /* output file */


        print()                                                              print
10      {
                int     lx, ly, firstgap, lastgap;     /* overlap */


                if ((fx = fopen(ofile, "w")) == 0) {
                        fprintf(stderr,"%s: can't write %s\n", prog, ofile);
15                      cleanup(1);
                }
                fprintf(fx, "<first sequence: %s (length = %d)\n", namex[0], len0);
                fprintf(fx, "<second sequence: %s (length = %d)\n", namex[1], len1);
                olen = 60;
20              lx = len0;
                ly = len1;
                firstgap = lastgap = 0;
                if (dmax < len1 - 1) {         /* leading gap in x */
                        pp[0].spc = firstgap = len1 - dmax - 1;
25                      ly -= pp[0].spc;
                }
                else if (dmax > len1 - 1) {    /* leading gap in y */
                        pp[1].spc = firstgap = dmax - (len1 - 1);
                        lx -= pp[1].spc;
30              }
                if (dmax0 < len0 - 1) {        /* trailing gap in x */
                        lastgap = len0 - dmax0 -1;
                        lx -= lastgap;
                }
35              else if (dmax0 > len0 - 1) {    /* trailing gap in y */
                        lastgap = dmax0 - (len0 - 1);
                        ly -= lastgap;
                }
                getmat(lx, ly, firstgap, lastgap);
40              pr_align();
        }
```

Page 1 of nwprint.c

```
45
        /*
         * trace back the best path, count matches
         */
        static
50      getmat(lx, ly, firstgap, lastgap)                                    getmat
                int     lx, ly;                /* "core" (minus endgaps) */
                int     firstgap, lastgap;     /* leading trailing overlap */
        {
                int     nm, i0, i1, siz0, siz1;
55              char    outx[32];
                double  pct;
                register        n0, n1;
                register char   *p0, *p1;
```

```
/* get total matches. score
 */
i0 = i1 = siz0 = siz1 = 0;
p0 = seqx[0] + pp[1].spc;
p1 = seqx[1] + pp[0].spc;
n0 = pp[1].spc + 1;
n1 = pp[0].spc + 1;

nm = 0;
while ( *p0 && *p1 ) {
        if (siz0) {
                p1++;
                n1++;
                siz0--;
        }
        else if (siz1) {
                p0++;
                n0++;
                siz1--;
        }
        else {
                if (xbm[*p0-'A']&xbm[*p1-'A'])
                        nm++;
                if (n0++ == pp[0].x[i0])
                        siz0 = pp[0].n[i0++];
                if (n1++ == pp[1].x[i1])
                        siz1 = pp[1].n[i1++];
                p0++;
                p1++;
        }
}

/* pct homology:
 * if penalizing endgaps, base is the shorter seq
 * else, knock off overhangs and take shorter core
 */
if (endgaps)
        lx = (len0 < len1)? len0 : len1;
else
        lx = (lx < ly)? lx : ly;
pct = 100.*(double)nm/(double)lx;
fprintf(fx, "\n");
fprintf(fx, "<%d match%s in an overlap of %d: %.2f percent similarity\n",
        nm, (nm == 1)? "" : "es", lx, pct);
```

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```
fprintf(fx, "<gaps in first sequence: %d", gapx);          ...getmat
if (gapx) {
        (void) sprintf(outx, " (%d %s%s)",
                ngapx, (dna)? "base":"residue", (ngapx == 1)? "":"s");
        fprintf(fx,"%s", outx);

fprintf(fx, ", gaps in second sequence: %d", gapy);
if (gapy) {
```

27

```
                    (void) sprintf(outx, " (%d %s%s)",
                            ngapy, (dna)? "base":"residue", (ngapy == 1)? "":"s");
                    fprintf(fx,"%s", outx);
            }
5       if (dna)
                    fprintf(fx,
                    "\n<score: %d (match = %d, mismatch = %d, gap penalty = %d + %d per base)\n",
                    smax, DMAT, DMIS, DINS0, DINS1);
        else
10                  fprintf(fx,
                    "\n<score: %d (Dayhoff PAM 250 matrix, gap penalty = %d + %d per residue)\n",
                    smax, PINS0, PINS1);
        if (endgaps)
                    fprintf(fx,
15                  "<endgaps penalized, left endgap: %d %s%s, right endgap: %d %s%s\n",
                    firstgap, (dna)? "base" : "residue", (firstgap == 1)? "" : "s",
                    lastgap, (dna)? "base" : "residue", (lastgap == 1)? "" : "s");
        else
                    fprintf(fx, "<endgaps not penalized\n");
20      }

        static          nm;             /* matches in core -- for checking */
        static          lmax;           /* lengths of stripped file names */
        static          ij[2];          /* jmp index for a path */
25      static          nc[2];          /* number at start of current line */
        static          ni[2];          /* current elem number -- for gapping */
        static          siz[2];
        static char     *ps[2];         /* ptr to current element */
        static char     *po[2];         /* ptr to next output char slot */
30      static char     out[2][P_LINE]; /* output line */
        static char     star[P_LINE];   /* set by stars() */


        /*
         * print alignment of described in struct path pp[]
35       */
        static
        pr_align()                                                                              pr_align
        {
                int             nn;     /* char count */
40              int             more;
                register        i;

                for (i = 0, lmax = 0; i < 2; i++) {
                        nn = stripname(namex[i]);
45                      if (nn > lmax)
                                lmax = nn;

                        nc[i] = 1;
                        ni[i] = 1;
50                      siz[i] = ij[i] = 0;
                        ps[i] = seqx[i];
                        po[i] = out[i];
                }
```

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28

```
            for (nn = nm = 0, more = 1; more; ) {                                   ...pr_align
                    for (i = more = 0; i < 2; i++) {
                            /*
5                           * do we have more of this sequence?
                            */
                            if (!*ps[i])
                                    continue;

10                          more++;

                            if (pp[i].spc) {        /* leading space */
                                    *po[i]++ = ' ';
                                    pp[i].spc--;
15                          }
                            else if (siz[i]) {      /* in a gap */
                                    *po[i]++ = '-';
                                    siz[i]--;
                            }
20                          else {                  /* we're putting a seq element
                                                    */
                                    *po[i] = *ps[i];
                                    if (islower(*ps[i]))
                                            *ps[i] = toupper(*ps[i]);
25                                  po[i]++;
                                    ps[i]++;

                                    /*
                                    * are we at next gap for this seq?
30                                  */
                                    if (ni[i] == pp[i].x[ij[i]]) {
                                            /*
                                            * we need to merge all gaps
                                            * at this location
35                                          */
                                            siz[i] = pp[i].n[ij[i]++];
                                            while (ni[i] == pp[i].x[ij[i]])
                                                    siz[i] += pp[i].n[ij[i]++];
                                    }
40                                  ni[i]++;
                            }
                    }
                    if (++nn == olen || !more && nn) {
                            dumpblock();
45                          for (i = 0; i < 2; i++)
                                    po[i] = out[i];
                            nn = 0;
                    }
            }
50  }

    /*
    * dump a block of lines, including numbers, stars: pr_align()
    */
55  static                                                                         dumpblock
    dumpblock()
    {
            register  i;
```

```
          for (i = 0; i < 2; i++)
                  *po[i]-- = '\0';
```

5

```
          (void) putc('\n', fx);
          for (i = 0; i < 2; i++) {
                  if (*out[i] && (*out[i] != ' ' || *(po[i]) != ' '))
                          if (i == 0)
                                  nums(i);
                          if (i == 0 && *out[1])
                                  stars();
                          putline(i);
                          if (i == 0 && *out[1])
                                  fprintf(fx, star);
                          if (i == 1)
                                  nums(i);
                  }
          }
  }

  /*
   * put out a number line: dumpblock()
   */
  static
  nums(ix)
          int       ix;        /* index in out[] holding seq line */
  {
          char               nline[P_LINE];
          register           i, j;
          register char      *pn, *px, *py;

          for (pn = nline, i = 0; i < lmax+P_SPC; i++, pn++)
                  *pn = ' ';
          for (i = nc[ix], py = out[ix]; *py; py++, pn++) {
                  if (*py == ' ' || *py == '-')
                          *pn = ' ';
                  else {
                          if (i%10 == 0 || (i == 1 && nc[ix] != 1)) {
                                  j = (i < 0)? -i : i;
                                  for (px = pn; j; j /= 10, px--)
                                          *px = j%10 + '0';
                                  if (i < 0)
                                          *px = '-';
                          }
                          else
                                  *pn = ' ';
                          i++;
                  }
          }
          *pn = '\0';
          nc[ix] = i;
          for (pn = nline; *pn; pn++)
                  (void) putc(*pn, fx);
          (void) putc('\n', fx);
  }

  /*
```

```
 * put out a line (name. [num]. seq. [num]): dumpblock()
 */
static
putline(ix)
         int      ix:
{



         int              i:
         register char    *px:

         for (px = namex[ix]. i = 0; *px && *px != ':'; px++. i++)
                  (void) putc(*px. fx);
         for (: i < lmax+P_SPC: i++)
                  (void) putc(' '. fx);

         /* these count from 1:
          * ni[] is current element (from 1)
          * nc[] is number at start of current line
          */
         for (px = out[ix]: *px: px++)
                  (void) putc(*px&0x7F. fx):
         (void) putc('\n'. fx):
}



/*
 * put a line of stars (seqs always in out[0]. out[1]): dumpblock()
 */
static
stars()
{
         int              i:
         register char    *p0. *p1. cx. *px;

         if (!*out[0] || (*out[0] == ' ' && *(po[0]) == ' ') ||
            !*out[1] || (*out[1] == ' ' && *(po[1]) == ' '))
                  return:
         px = star;
         for (i = lmax+P_SPC; i; i--)
                  *px++ = ' ';

         for (p0 = out[0]. p1 = out[1]; *p0 && *p1; p0++. p1++){
                  if (isalpha(*p0) && isalpha(*p1)){

                           if (xbm[*p0-'A']&xbm[*p1-'A'])
                                    cx = '*';
                                    nm++;
                           }
                           else if (!dna && _day[*p0-'A'][*p1-'A'] > 0)
                                    cx = '.';
                           else
                                    cx = ' ';
                  }
                  else
                           cx = ' ';
                  *px++ = cx:
```

```
                }
                *px++ = '\n':
                *px = '\0':
        }
```
5

10
```
        /*
         * strip path or prefix from pn, return len: pr_align()
         */
        static
        stripname(pn)                                                              stripname
                char    *pn;     /* file name (may be path) */
        {
                register char    *px, *py;

20
                py = 0:
                for (px = pn; *px; px++)
                        if (*px == '/')
                                py = px + 1;
                if (py)
                        (void) strcpy(pn, py);
                return(strlen(pn));

        }
```

30

35
```
        /*
         * cleanup() -- cleanup any tmp file
         * getseq() -- read in seq, set dna, len, maxlen
         * g_calloc() -- calloc() with error checkin
         * readjmps() -- get the good jmps, from tmp file if necessary
         * writejmps() -- write a filled array of jmps to a tmp file: nw()
         */
        #include "nw.h"
        #include <sys/file.h>

        char    *jname = "/tmp/homgXXXXXX";       /* tmp file for jmps */
        FILE    *fj;

        int     cleanup();                         /* cleanup tmp file */
        long    lseek();
50
        /*
         * remove any tmp file if we blow
         */
        cleanup(i)                                                                 cleanup
                int     i;
        {
                if (fj)
                        (void) unlink(jname);
                exit(i);
```

```
        }

        /*
         * read. return ptr to seq. set dna. len. maxlen
 5       * skip lines starting with ':'. '<'. or '>'
         * seq in upper or lower case
         */
        char    *
        getseq(file, len)                                                           getseq
10              char    *file:        /* file name */
                int     *len:         /* seq len */

        {
                char            line[1024]. *pseq:
                register char   *px. *py:
15              int             natgc. tlen:
                FILE            *fp:

                if ((fp = fopen(file."r")) == 0) {
                        fprintf(stderr."%s: can't read %s\n". prog. file):
20                      exit(1):
                }
                tlen = natgc = 0:
                while (fgets(line. 1024. fp)) {
                        if (*line == ':' || *line == '<' || *line == '>')
25                              continue:
                        for (px = line: *px != '\n': px++)
                                if (isupper(*px) || islower(*px))
                                        tlen++:
                }
30              if ((pseq = malloc((unsigned)(tlen+6))) == 0) {
                        fprintf(stderr."%s: malloc() failed to get %d bytes for %s\n". prog. tlen+6. file):
                        exit(1):
                }
                pseq[0] = pseq[1] = pseq[2] = pseq[3] = '\0':
35
```

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```
40
                py = pseq + 4:
                *len = tlen;
                rewind(fp):

45              while (fgets(line. 1024. fp)) {
                        if (*line == ':' || *line == '<' || *line == '>')
                                continue;
                        for (px = line; *px != '\n': px++){
                                if (isupper(*px))
50                                      *py++ = *px:
                                else if (islower(*px))
                                        *py++ = toupper(*px):
                                if (index("ATGCU".*(py-1)))
                                        natgc++:
55                      }
                }
                *py++ = '\0':
                *py = '\0';
                (void) fclose(fp):
```

```
          dna = natgc > (tlen/3);
          return(pseq+4);
     }


5    char      *
     g_calloc(msg, nx, sz)                                                              g_calloc
          char      *msg;              /* program, calling routine */
          int       nx, sz;            /* number and size of elements */
     {
10        char           *px, *calloc();

          if ((px = calloc((unsigned)nx, (unsigned)sz)) == 0) {
               if (*msg) {
                    fprintf(stderr, "%s: g_calloc() failed %s (n=%d, sz=%d)\n", prog, msg, nx, sz);
15                  exit(1);
               }
          }
          return(px);
     }
20
     /*
      * get final jmps from dx[] or tmp file, set pp[], reset dmax; main()
      */
     readjmps()                                                                         readjmps
25   {
          int            fd = -1;
          int            siz, i0, i1;
          register  i, j, xx;

30        if (fj) {
               (void) fclose(fj);
               if ((fd = open(jname, O_RDONLY, 0)) < 0) {
                    fprintf(stderr, "%s: can't open() %s\n", prog, jname);
                    cleanup(1);
35             }
          }
          for (i = i0 = i1 = 0, dmax0 = dmax, xx = len0; ; i++) {
               while (1) {
                    for (j = dx[dmax].ijmp; j >= 0 && dx[dmax].jp.x[j] >= xx; j--)
40                       ;
```

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```
                    if (j < 0 && dx[dmax].offset && fj) {
45                       (void) lseek(fd, dx[dmax].offset, 0);
                         (void) read(fd, (char *)&dx[dmax].jp, sizeof(struct jmp));
                         (void) read(fd, (char *)&dx[dmax].offset, sizeof(dx[dmax].offset));
                         dx[dmax].ijmp = MAXJMP-1;
                    }
50                  else
                         break;
               }
               if (i >= JMPS) {
                    fprintf(stderr, "%s: too many gaps in alignment\n", prog);
55                  cleanup(1);
               }
               if (j >= 0) {
                    siz = dx[dmax].jp.n[j];
```

```
                    xx = dx[dmax].jp.x[j]:
                    dmax += siz:
                    if (siz < 0) {                    /* gap in second seq */
                            pp[1].n[i1] = -siz:
  5                         xx += siz:

                            /* id = xx - yy + len1 - 1
                             */
                            pp[1].x[i1] = xx - dmax + len1 - 1:
 10                         gapy++:
                            ngapy -= siz:
    /* ignore MAXGAP when doing endgaps */
                            siz = (-siz < MAXGAP || endgaps)? -siz : MAXGAP:
                            i1++:
 15                 }
                    else if (siz > 0) {    /* gap in first seq */
                            pp[0].n[i0] = siz:
                            pp[0].x[i0] = xx:
                            gapx++:
 20                         ngapx += siz:
    /* ignore MAXGAP when doing endgaps */
                            siz = (siz < MAXGAP || endgaps)? siz : MAXGAP:
                            i0++:
                    }
 25             }
                    else
                            break:
            }

 30         /* reverse the order of jmps
             */
            for (j = 0, i0--; j < i0; j++, i0--) {
                    i = pp[0].n[j]; pp[0].n[j] = pp[0].n[i0]; pp[0].n[i0] = i:
                    i = pp[0].x[j]; pp[0].x[j] = pp[0].x[i0]; pp[0].x[i0] = i:
 35         }
            for (j = 0, i1--; j < i1; j++, i1--) {
                    i = pp[1].n[j]; pp[1].n[j] = pp[1].n[i1]; pp[1].n[i1] = i:
                    i = pp[1].x[j]; pp[1].x[j] = pp[1].x[i1]; pp[1].x[i1] = i:
            }
 40         if (fd >= 0)
                    (void) close(fd):
            if (fj) {
                    (void) unlink(jname):
                    fj = 0:
 45                 offset = 0;
            }
    }                                                          Page 3 of nwsubr.c


 50 /*
     * write a filled jmp struct offset of the prev one (if any): nw()
     */
    writejmps(ix)                                                              writejmps
            int      ix:
 55 {
            char     *mktemp():

            if (!fj) {  .
                    if (mktemp(jname) < 0) {
 60                         fprintf(stderr. "%s: can't mktemp() %s\n", prog, jname):
```

```
                              cleanup( 1 );
                       }
                       if ((fj = fopen(jname, "w")) == 0) {
                              fprintf( stderr, "%s: can't write %s\n", prog, jname);
5                             exit( 1 );
                       }
                }
                (void) fwrite((char *)&dx[ix].jp, sizeof(struct jmp), 1, fj);
                (void) fwrite((char *)&dx[ix].offset, sizeof(dx[ix].offset), 1, fj);
10       }
```

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For purposes herein. the % nucleic acid sequence identity of a given nucleic acid sequence C to. with. or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with. or against a given nucleic acid sequence D) is calculated as follows:

5   100 times the fraction W/Z

where W is the number of nucleotides scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of C and D. and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

10   Unless specifically stated otherwise. all % nucleic acid sequence identity values used herein are obtained as described above using the ALIGN-2 sequence comparison computer program. However. % nucleic acid sequence identity may also be determined using the sequence comparison program NCBI-BLAST2 (Altschul *et al..* Nucleic Acids Res., 25:3389-3402 (1997)). The NCBI-BLAST2 sequence comparison program may be downloaded from http://www.ncbi.nlm.nih.gov. NCBI-BLAST2 uses several search parameters. wherein all of those search

15   parameters are set to default values including. for example. unmask = yes. strand = all, expected occurrences = 10. minimum low complexity length = 15/5. multi-pass e-value = 0.01. constant for multi-pass = 25. dropoff for final gapped alignment = 25 and scoring matrix = BLOSUM62.

In situations where NCBI-BLAST2 is employed for sequence comparisons, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can

20   alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows:

100 times the fraction W/Z

where W is the number of nucleotides scored as identical matches by the sequence alignment program NCBI-BLAST2 in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be

25   appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D. the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

In addition. % nucleic acid sequence identity values may also be generated using the WU-BLAST-2 computer program (Altschul *et al.,* Methods in Enzymology. 266:460-480 (1996)). Most of the WU-BLAST-2 search parameters are set to the default values. Those not set to default values, *i.e.,* the adjustable parameters, are

30   set with the following values: overlap span = 1. overlap fraction = 0.125. word threshold (T) = 11. and scoring matrix = BLOSUM62. For purposes herein. a % nucleic acid sequence identity value is determined by dividing (a) the number of matching identical nucleotides between the nucleic acid sequence of the LIV-1 polypeptide-encoding nucleic acid molecule of interest having a sequence derived from the native sequence LIV-1 polypeptide-encoding nucleic acid and the comparison nucleic acid molecule of interest (*i.e..* the sequence against which the

35   LIV-1 polypeptide-encoding nucleic acid molecule of interest is being compared which may be a variant LIV-1 polynucleotide) as determined by WU-BLAST-2 by (b) the total number of nucleotides of the LIV-1 polypeptide-encoding nucleic acid molecule of interest. For example, in the statement "an isolated nucleic acid molecule

37

comprising a nucleic acid sequence A which has or having at least 80% nucleic acid sequence identity to the nucleic acid sequence B", the nucleic acid sequence A is the comparison nucleic acid molecule of interest and the nucleic acid sequence B is the nucleic acid sequence of the LIV-1 polypeptide-encoding nucleic acid molecule of interest.

5   The term "positives", in the context of the amino acid sequence identity comparisons performed as described above, includes amino acid residues in the sequences compared that are not only identical, but also those that have similar properties. Amino acid residues that score a positive value to an amino acid residue of interest are those that are either identical to the amino acid residue of interest or are a preferred substitution (as defined in Table 2 below) of the amino acid residue of interest.

10   Table 2

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro; ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	leu
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in function or immunological identity of the polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro: and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into

5   the remaining (non-conserved) sites.

The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter *et al.*, Nucl. Acids Res., 13:4331 (1986): Zoller *et al.*, Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells *et al.*, Gene, 34:315 (1985)], restriction selection mutagenesis [Wells *et al.*, Philos. Trans. R. Soc. London SerA, 317:415 (1986)]

10   or other known techniques can be performed.

For purposes herein, the % value of positives of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % positives to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

15   where X is the number of amino acid residues scoring a positive value as defined above by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % positives of A to B will not equal the % positives of B to A.

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has

20   been identified and separated and/or recovered from a component of its natural environment. Preferably, the isolated polypeptide is free of association with all components with which it is naturally associated. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15

25   residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the LIV-1 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

30   An "isolated" nucleic acid molecule encoding a LIV-1 polypeptide or an "isolated" nucleic acid encoding an anti-LIV-1 antibody, is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the LIV-1-encoding nucleic acid or the anti-LIV-1-encoding nucleic acid. Preferably, the isolated nucleic acid is free of association with all components with which it is naturally associated. An isolated LIV-1-encoding nucleic acid molecule or an anti-

35   LIV-1-encoding nucleic acid molecule is other than in the form or setting in which it is found in nature.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example,

include a promoter, optionally an operator sequence. and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide

5   if it is expressed as a preprotein that participates in the secretion of the polypeptide: a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence: or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally. "operably linked" means that the DNA sequences being linked are contiguous. and, in the case of a secretory leader. contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at

10   convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art. and generally is an empirical calculation dependent upon probe length, washing temperature. and salt concentration. In general, longer probes require higher temperatures for proper annealing. while shorter probes need lower

15   temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent. while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions. see Ausubel

20   et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers. (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent. such as formamide. for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1%

25   polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride. 75 mM sodium citrate at 42°C: or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl. 0.075 M sodium citrate). 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution. sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

30   "Moderately stringent conditions" may be identified as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and % SDS) less stringent than those described above. An example of moderately stringent conditions is overnight incubation at 37°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate). 50 mM sodium phosphate (pH 7.6). 5 x Denhardt's

35   solution. 10% dextran sulfate, and 20 mg/ml denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 35-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength. etc. as necessary to accommodate factors such as probe length and the like.

"Active" or "activity" for the purposes herein refers to form(s) of LIV-1 polypeptides which retain a
biological and/or an immunological activity/property of a native or naturally-occurring LIV-1 polypeptide, wherein
"biological" activity refers to a function (either inhibitory or stimulatory) caused by a native or naturally-occurring
LIV-1 polypeptide other than the ability to induce the production of an antibody against an antigenic epitope
5   possessed by a a native or naturally-occurring LIV-1 polypeptide and an "immunological" activity refers to the
ability to induce the production of an antibody against an antigenic epitope possessed by a native or naturally-
occurring LIV-1 polypeptide.

"Biological activity" in the context of an antibody or another antagonist molecule that can be identified by
the screening assays disclosed herein (e.g., an organic or inorganic small molecule, peptide, etc.) is used to refer
10   to the ability of such molecules to bind or complex with the polypeptides encoded by the amplified genes identified
herein, or otherwise interfere with the interaction of the encoded polypeptides with other cellular proteins or
otherwise interfere with the transcription or translation of a LIV-1 polypeptide. A preferred biological activity is
growth inhibition of a target tumor cell. Another preferred biological activity is cytotoxic activity resulting in the
death of the target tumor cell.

15   The term "biological activity" in the context of a LIV-1 polypeptide means the ability of a LIV-1
polypeptide to induce neoplastic cell growth or uncontrolled cell growth or to act as an indication of a particular
form of neoplasm that is particularly metastatic.

The phrase "immunological activity" means immunological cross-reactivity with at least one epitope of
a LIV-1 polypeptide.

20   "Immunological cross-reactivity" as used herein means that the candidate polypeptide is capable of
competitively inhibiting the qualitative biological activity of a LIV-1 polypeptide having this activity with
polyclonal antisera raised against the known active LIV-1 polypeptide. Such antisera are prepared in conventional
fashion by injecting goats or rabbits, for example, subcutaneously with the known active analogue in complete
Freund's adjuvant, followed by booster intraperitoneal or subcutaneous injection in incomplete Freunds. The
25   immunological cross-reactivity preferably is "specific", which means that the binding affinity of the
immunologically cross-reactive molecule (e.g., antibody) identified, to the corresponding LIV-1 polypeptide is
significantly higher (preferably at least about 2-times, more preferably at least about 4-times, even more preferably
at least about 8-times, most preferably at least about 10-times higher) than the binding affinity of that molecule to
any other known native polypeptide.

30   The term "antagonist" is used in the broadest sense, and includes any molecule that partially or fully blocks,
inhibits, or neutralizes a biological activity of a native LIV-1 polypeptide disclosed herein or the transcription or
translation thereof. Suitable antagonist molecules specifically include antagonist antibodies or antibody fragments,
fragments, peptides, small organic molecules, anti-sense nucleic acids, etc. Included are methods for identifying
antagonists of a LIV-1 polypeptide with a candidate antagonist molecule and measuring a detectable change in one
35   or more biological activities normally associated with the LIV-1 polypeptide.

A "small molecule" is defined herein to have a molecular weight below about 500 Daltons.

The word "label" when used herein refers to a detectable compound or composition which is conjugated

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directly or indirectly to the antibody so as to generate a "labeled" antibody. The label may be detectable by itself (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable. Radionuclides that can serve as detectable labels include, for example, I-131, I-123, I-125, Y-90, Re-188, Re-186, At-211, Cu-67, Bi-212, and Pd-109.

5   By "solid phase" is meant a non-aqueous matrix to which the antibody of the present invention can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid

10   phase of discrete particles, such as those described in U.S. Patent No. 4,275,149.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as a LIV-1 polypeptide or antibody thereto and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

15   As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous protein (an "adhesin," for example a receptor, ligand, or enzyme) with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of the adhesin amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site (antigen combining site) of an antibody (i.e., is "heterologous"), and an immunoglobulin constant domain

20   sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM, and any subclass or isotype thereof.

The terms "HER2", "ErbB2" "c-Erb-B2" are used interchangeably. Unless indicated otherwise, the terms

25   "ErbB2" "c-Erb-B2" and "HER2" when used herein refer to the human protein, and "erbB2," "c-erb-B2," and "her2" refer to human gene. The human erbB2 gene and ErbB2 protein are, for example, described in Semba et al., PNAS (USA) 82:6497-6501 (1985) and Yamamoto et al. Nature 319:230-234 (1986) (Genebank accession number X03363). ErbB2 comprises four domains (Domains 1-4).

The "epitope 4D5" is the region in the extracellular domain of ErbB2 to which the antibody 4D5 (ATCC

30   CRL 10463) binds. This epitope is close to the transmembrane region of ErbB2. To screen for antibodies which bind to the 4D5 epitope, a routine cross-blocking assay such as that described in Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed to assess whether the antibody binds to the 4D5 epitope of ErbB2 (i.e. any one or more residues in the region from about residue 529, e.g. about residue 561 to about residue 625, inclusive).

35   The "epitope 3H4" is the region in the extracellular domain of ErbB2 to which the antibody 3H4 binds. This epitope includes residues from about 541 to about 599, inclusive, in the amino acid sequence of ErbB2 extracellular domain.

The "epitope 7C2/7F3" is the region at the N terminus of the extracellular domain of ErbB2 to which the 7C2 and/or 7F3 antibodies (ATCC HB-12215 and ATCC HB-12216. respectively) bind. To screen for antibodies which bind to the 7C2/7F3 epitope. a routine cross-blocking assay such as that described in *Antibodies. A Laboratory Manual*, Cold Spring Harbor Laboratory. Ed Harlow and David Lane (1988). can be performed. Alternatively, epitope mapping can be performed to establish whether the antibody binds to the 7C2/7F3 epitope on ErbB2.

The term "induces cell death" or "capable of inducing cell death" refers to the ability of the anti-LIV-1 gene product antibody. alone or in co-treatment with a chemotherapeutic agent, to make a viable cell become nonviable. The "cell" here is one which expresses the LIV-1 gene product, especially where the cell overexpresses the LIV-1 gene product. A cell which "overexpresses" LIV-1 has significantly higher than normal LIV-1 mRNA and/or LIV-1 protein levels compared to a noncancerous cell of the same tissue type. A cell to be treated by the method of the invention does not also overexpress ErbB2 (i.e. the cell expresses ErbB2 at a level that is approximately the same or less than a normal, non-cancerous cell of the same cell or tissue type). Preferably. the cell is a cancer cell, *e.g.* a breast, lung, or prostate cell. *In vitro*. the cell may be from a cell line transformed with LIV-1 DNA, preferably DNA164647, to express LIV-1 on the cell surface. Cell death *in vitro* may be determined in the absence of complement and immune effector cells to distinguish cell death induced by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC). Thus, the assay for cell death may be performed using heat inactivated serum (*i.e.* in the absence of complement) and in the absence of immune effector cells. To determine whether the antibody is able to induce cell death, loss of membrane integrity as evaluated by uptake of propidium iodide (PI), trypan blue (see Moore *et al. Cytotechnology* 17:1-11 [1995]) or 7AAD can be assessed relative to untreated cells. Preferred cell death-inducing antibodies are those which induce PI uptake in the "PI uptake assay in LIV-1 expressing cells".

The phrase "induces apoptosis" or "capable of inducing apoptosis" refers to the ability of the antibody to induce programmed cell death as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation. and/or formation of membrane vesicles (called apoptotic bodies). The cell is one which overexpresses the LIV-1 gene product. Preferably the "cell" is a tumor cell. *e.g.* a breast, lung, or prostate cell. *In vitro*, the cell may be from a cell line transformed with LIV-1 DNA, such as DNA164647. Various methods are available for evaluating the cellular events associated with apoptosis. For example, phosphatidyl serine (PS) translocation can be measured by annexin binding; DNA fragmentation can be evaluated through DNA laddering as disclosed in the example herein; and nuclear/chromatin condensation along with DNA fragmentation can be evaluated by any increase in hypodiploid cells. Preferably, the antibody which induces apoptosis is one which results in about 2 to 50 fold, preferably about 5 to 50 fold, and most preferably about 10 to 50 fold induction of annexin binding relative to untreated cell in an "annexin binding assay using cells" (see below).

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG_1, IgG_2, IgG_3, or IgG_4) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with the anti-LIV-1 gene product antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include benign and malignant tumors of breast, lung, and prostate tissue.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

The term "serum concentration," "serum drug concentration," or "serum anti-LIV-1 antibody concentration" refers to the concentration of a drug in the blood serum of an animal or human patient being treated with the drug. Serum concentration of antibody, for example, is preferably determined by immunoassay. Preferably, the immunoassay is ELISA according to the procedure disclosed herein.

The term "peak serum concentration" refers to the maximal serum drug concentration shortly after delivery of the drug into the animal or human patient, after the drug has been distributed throughout the blood system, but before significant tissue distribution, metabolism or excretion of drug by the body has occurred.

The term "trough serum concentration" refers to the serum drug concentration at a time after delivery of a previous dose and immediately prior to delivery of the next subsequent dose of drug in a series of doses. Generally, the trough serum concentration is a minimum sustained efficacious drug concentration in the series of drug administrations. Also, the trough serum concentration is frequently targeted as a minimum serum concentration for efficacy because it represents the serum concentration at which another dose of drug is to be administered as part of the treatment regimen. If the delivery of drug is by intravenous administration, the trough serum concentration is most preferably attained within 1 day of a front loading initial drug delivery. If the delivery of drug is by subcutaneous administration, the peak serum concentration is preferably attained in 3 days or less. According to the invention, the trough serum concentration is preferably attained in 4 weeks or less, preferably 3 weeks or less, more preferably 2 weeks or less, most preferably in 1 week or less, including 1 day or less using any of the drug delivery methods disclosed herein.

The term "intravenous infusion" refers to introduction of a drug into the vein of an animal or human patient over a period of time greater than approximately 5 minutes, preferably between approximately 30 to 90 minutes, although, according to the invention, intravenous infusion is alternatively administered for 10 hours or less.

The term "intravenous bolus" or "intravenous push" refers to drug administration into a vein of an animal or human such that the body receives the drug in approximately 15 minutes or less, preferably 5 minutes or less.

The term "subcutaneous administration" refers to introduction of a drug under the skin of an animal or human patient, preferable within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle. The pocket may be created by pinching or drawing the skin up and away from underlying tissue.

The term "subcutaneous infusion" refers to introduction of a drug under the skin of an animal or human

patient, preferably within a pocket between the skin and underlying tissue, by relatively slow, sustained delivery from a drug receptacle for a period of time including, but not limited to, 30 minutes or less, or 90 minutes or less. Optionally, the infusion may be made by subcutaneous implantation of a drug delivery pump implanted under the skin of the animal or human patient, wherein the pump delivers a predetermined amount of drug for a predetermined

5   period of time, such as 30 minutes, 90 minutes, or a time period spanning the length of the treatment regimen.

The term "subcutaneous bolus" refers to drug administration beneath the skin of an animal or human patient, where bolus drug delivery is preferably less than approximately 15 minutes, more preferably less than 5 minutes, and most preferably less than 60 seconds. Administration is preferably within a pocket between the skin and underlying tissue, where the pocket is created, for example, by pinching or drawing the skin up and away from

10   underlying tissue.

The term "front loading" when referring to drug administration is meant to describe an initially higher dose followed by the same or lower doses at intervals. The initial higher dose or doses are meant to more rapidly increase the animal or human patient's serum drug concentration to an efficacious target serum concentration.

Published information related to LIV-1 gene expression and gene product includes the following issued

15   patents and published applications: Manning, D.L. et al., U.S. Patent No. 5,693,465, issued December 2, 1997; Manning, D.L. et al., European J. Cancer 29A(10):1462-1468 [1993]; Manning, D.L. et al., European J. Cancer. 30A(5):675-678 [1994]; Manning, D.L. et al., Acta Oncologica 34(5):641-646 [1995];McClelland, R.A. et al., Breast Cancer Res. & Treatment 41(1):31-41 [1996]; Knowlden, J.M. et al., Clin. Cancer Res. 3(11):2165-2172 [1997]; and McClelland, R.A., et al., British J. Cancer 77(10):1653-1656 [1998].

20   Published information related to anti-ErbB2 antibody includes the following issued patents and published applications: PCT/US89/00051, published January 5, 1989, PCT/US90/02697, published May 18, 1990, EU 0474727, issued July 23, 1997, DE 69031120.6, issued July 23, 1997, PCT/US97/18385, published October 9, 1997, SA 97/9185, issued October 14, 1997, US 5677171, issued October 14, 1997, US 5720937, issued February 24, 1998, US 5720954, issued February 24, 1998, US 5725856, issued March 10, 1998, US 5770195, issued June

25   23, 1998, US 5772997, issued June 30, 1998, PCT/US98/2626, published December 10, 1998, and PCT/US99/06673, published March 26, 1999, each of which patents and publications is herein incorporated by reference in its entirety.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

30   All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and

35   throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, VA. Unless otherwise noted, the present invention uses standard procedures of recombinant DNA technology, such as those described herein and in the following textbooks: Sambrook *et al.*, *Molecular Cloning: A Laboratory*

Manual. Cold Spring Harbor Press N.Y.. 1989: Ausubel *et al.. Current Protocols in Molecular Biology.* Green
Publishing Associates and Wiley Interscience. N.Y.. 1989: Innis *et al.. PCR Protocols: A Guide to Methods and
Applications,* Academic Press, inc.. N.Y., 1990: Harlow *et al., Antibodies: A Laboratory Manual.* Cold Spring
Harbor Press, Cold Spring Harbor. 1988: Gait. M.J.. *Oligonucleotide Synthesis.* IRL Press. Oxford. 1984: R.I.
5   Freshney. *Animal Cell Culture,* 1987: Coligan *et al.. Current Protocols in Immunology.* 1991.

Example 1.   LIV-1 Expression in Tumor Cells Examined by Microarray Analysis

A form of breast cancer in which the cells overexpress the LIV-1 gene product (e.g. LIV-1-164647 mRNA)
but do not overexpress ErbB2 has been discovered and is uniquely disclosed herein. Detection of the tumor type
was made using microarray technology. Using nucleic acid microarrays. test and control mRNA samples from test
10   and control tissue samples are reverse transcribed and labeled to generate cDNA probes. The cDNA probes are then
hybridized to an array of nucleic acids immobilized on a solid support. The array is configured such that the
sequence and position of each member of the array is known. Hybridization of a labeled probe with a particular
array member indicates that the sample from which the probe was derived expresses that gene. If the hybridization
signal of a probe from a test (disease tissue) sample is greater than hybridization signal of a probe from a control
15   (normal tissue) sample, the gene or genes overexpressed in the disease tissue are identified. The implication of this
result is that an overexpressed protein in a diseased tissue is useful not only as a diagnostic marker for the presence
of the disease condition, but also as a therapeutic target for treatment of the disease condition.

Tumor cells were grossly dissected from surrounding. non-cancerous cells in breast tumor tissue.
Hematoxylin and eosin staining of the cells confirmed that the excised cells were from tumor. The mRNA of the
20   tumor cells (reflecting cell-specific expression of a variety of genes) was converted to cDNA by RT/PCR
methodology, labeled with fluorescent tags, and allowed to hybridize to the ESTs arrayed on a glass slide. An
imaging device detected and measured the fluorescence of each sample on the slide. where fluorescence represents
a labeled messenger from the test cells identifiable due to its hybridization with a known nucleic acid sequence (an
EST) at a known position on the slide. Relative fluorescence indicated relative activity of a gene. with strong
25   flourescence indicating an active gene expressing a relative large amount of messenger. Little or no flourescence
indicated that no labeled messenger hybridized to the ESTs. Detection of fluorescently labeled probes hybridized
to sequences on the microarray slide is described. for example. in U.S. Patent 5.143,854. herein incorporated by
reference.

It was found that cDNA of the preparation hybridized to a publicly available EST sequence (accession no.
30   H293l5 (from the Washington University-Merck EST Project, authors Hillier, L. et al., SEQ ID NO:5, purchased
from Research Genetics (Alabama. USA) in a pattern suggesting overexpression in breast tumor tissue that did not
also overexpress ErbB2. The cDNA was sequenced and disclosed herein as LIV-1-DNA164647. *In situ*
hybridization of a radioactively labeled LIV-1-164647 probe to a tissue microarray of tumor tissues further indicated
LIV-1 expression in tumors from breast. lung. prostate, colon, endometrial, ovarian. and transitional carcinomas,
35   and melanoma tissue samples.

It was further discovered that breast tumor cells which overexpressed LIV-1 did not overexpress ErbB2
relative to a normal cell of the same type (where a normal cell refers to a cell that is not cancerous and free of any

other disease in which LIV-1 may be overexpressed). The very low expression of LIV-1 in normal tissues relative to the overexpression of LIV-1 in breast tumor cells makes LIV-1 a desirable target for a therapeutic antibody.

For example, in breast tumors, strong fluorescent detection of LIV-1 occurred with low detection of ErbB2 expression, or alterntively strong flourescent detection of ErbB2 was observed in the absence of LIV-1 mRNA

5　expression. Of 17 samples, each from a different breast tumor of variable tumor cell content, 6 samples showed moderate to strong LIV-1 expression, and 6 showed moderate or strong ErbB2 expression. There was an overlap in one tumor which showed moderate ErbB2 and LIV-1 expression. In another tumor it appeared that strong detection of ErbB2 expression was accompanied by very weak detection of LIV-1 expression. Cell lines expressing endogenous or exogenous LIV-1 protein (preferably LIV-1-164647), but not overexpressing ErbB2, are thus useful

10　for testing the detection or LIV-1 expression relative to ErbB2 expression. Such cells are also useful to test anti-LIV-1 antibodies for binding to LIV-1-164647-expressing cells and their affects on cell growth.

Regions of LIV-1-164647 in the ECD and in the C-terminus were found to be unique as compared to the LIV-1 sequence of Green et al. (see Fig. 4 comparing SEQ ID NO:4 and GenBank Accession No. AAA96258). The region spanned amino acids 126 to and including amino acid 132 of SEQ ID NO:4 (SEQ ID NO:17) and amino

15　acids 743 to 755 (SEQ ID NO:18). A larger histidine-rich region from amino acid 114 to and including amino acid 135 (SEQ ID NO:19) is also encompassed by the invention. Thus, according to the presently disclosed invention, the nucleic acid of the invention comprises a sequence having at least 65%, preferably at least 75%, more preferably at least 85%, still more preferably at least 90%, and most preferably at least 96% homologous to a sequence from approximately nucleotide 412 to and including nucleotide 477 of SEQ ID NO:3, or its complementary sequence.

20　Preferably, the nucleic acid of the invention comprises a sequence from nucleotide 443 to and including nucleic acid 446 of SEQ ID NO:3. The present invention further includes an isolated polypeptide comprising an amino acid sequence having at least 65%, preferably at least 75%, more preferably at least 85%, still more preferably at least 90%, and most preferably at least 96% homology to a sequence from approximately amino acid 114 to and including amino acid 135 of SEQ ID NO:4. Preferably, the isolated polypeptide of the invention comprises an amino acid

25　sequence from amino acid 126 to and including amino acid 132 of SEQ ID NO:4.

Example 2. __Preparation of the LIV-1 polypeptides__

A previously unknown LIV-1 gene and its encoded protein are uniquely disclosed herein. The nucleic acid sequence of LIV-1 (DNA164647; SEQ ID NO:3 and its complement) and amino acid sequence of LIV-1 encoded therein (SEQ ID NO:4) are uniquely disclosed herein. This example describes the preparation and isolation of the

30　presently disclosed LIV-1 protein encoded by DNA164647. Subsequent examples describe the tissue expression profile of LIV-1 and its partial exposure at the cell surface. The LIV-1 gene is disclosed herein to be expressed in various tumor tissues including breast, lung, prostate, and colon. Cell surface expression of this tumor-associated protein makes LIV-1 a useful target for cancer detection and treatment.

The LIV-1 described by DNA164647 has a unique nucleic acid sequence and unique amino acid sequence.

35　The presently disclosed LIV-1 differs in nucleic acid sequence and amino acid sequence from another form of LIV-1 protein previously described by Green et al. (see Green, C. et al., direct submission, GenBank Accession Nos. U41060 (nucleic acid sequence) and AAA96258 (amino acid sequence)). The nucleic acid sequence of AAA96258

LIV-1 (SEQ ID NO:1 (coding sequence)) is shown in Fig. 1A. The predicted amino acid sequence (AAA96258; SEQ ID NO:2) encoded by nucleic acid sequence U41061 (SEQ ID NO:1) is shown in Fig. 1B. Included in the diagram are predictions for sequences encoding a signal sequence, extracellular domain. and transmembrane domain. Significantly, the presently disclosed LIV-1 (DNA164647) differs in predicted extracellular domain region.

5   The present invention provides methods for using DNA164647 encoding LIV-1 polypeptide for the production of compounds inhibiting neoplastic growth as well as for the preparation of screening methods for identifying growth inhibitory compounds (e.g. tumor compounds). In particular, cDNAs encoding certain LIV-1 polypeptides. For the sake of simplicity, in the present specification the proteins encoded by nucleic acid referred to as "DNA164647", as well as all further native homologues and variants included in the foregoing definition of

10   LIV-1 polypeptide, will be referred to as "LIV-1" polypeptide, regardless of their origin or mode of expression.

Cloning of DNA164647 LIV-1 nucleic acid: Full Length Inverse PCR ("FLIP"), also referred to as inverse long distance PCR because of the ability of this method to isolate long genes. was used to clone DNA164647 LIV-1 nucleic acid. The FLIP PCR technique is described in pending U.S. application 09/480.782. filed January 10, 2000, J.C. Grimaldi et al (inventors). Genentech, Inc. (assignee), which application is herein incorporated by reference

15   in its entirety, particularly with respect to the method of cloning a gene. While standard cloning techniques may be used to clone full length genes, FLIP is a very rapid and high-throughput method of isolating an entire clone, vector plus insert, of a specific nucleic acid molecule from any nucleic acid library which was propagated in a host cell that methylates the nucleic acid library. The FLIP cloning method amplifies a target gene or nucleotide sequence and generates a highly purified population of the target gene. Fig. 5 is a flow diagram of a FLIP cloning

20   process. DNA164647 was isolated by FLIP methodology using the following primers and probe.

forward primer: LIV1-INV5' 5'ATGTTGACTTGGCAATTTCCACACGGCA 3' (SEQ ID NO:6)
reverse primer: LIV1-INV3' 5'TAATGCCAGATTCCCAATTCGGACTAA 3' (SEQ ID NO:7)
probe: LIV1-p   5'TTAGTTCATGAAGGGGATTTGTGACAGAGAGGGCAAAGGTCAGGAT 3' (SEQ ID

25   NO:8).

           A human LIV-1 gene (Genbank Accession# U41060) has been sequenced by C. Green et al. (direct submission, November 21, 1995). The sequence is 3461 bp (SEQ ID NO:1; Fig. 1A) and includes the an open reading frame (ORF). Using the FLIP methodology and the primers and probe disclosed above, a cDNA clone was isolated from a pool of fifty human cDNAs from cDNA libraries representing various tissues (Genentech cDNA

30   libraries, Genentech, Inc., So. San Francisco, CA). The isolated cDNA clone includes a LIV-1 gene comprising a variant nucleic acid sequence (DNA164647; SEQ ID NO:3 (coding sequence) and its complementary sequence) and a variant deduced amino acid sequence (SEQ ID NO:4). The total length of the isolated LIV-1 DNA164674 nucleic acid is 2776 bp; the vector pRK5D used was 5.1 kb, thus adding to a total length of 7.9 kb of the DNA molecule amplified by IPCR and isolated.

35   A nucleic acid sequence comparison of the LIV-1 submitted by Green et al., *supra*. and DNA164647 disclosed herein is shown in Fig. 3. Asterisks denote identity between nucleotides. while dashes represent gaps where no identity occurs. An insertion of 18 nucleotides is observed at approximately nucleotides 446 to 463 of

DNA 164647 (SEQ ID NO:3) and two single nucleotide insertions occur at nucleotides 2297 and 2323 causing a frame shift and different deduced amino acid sequences in that region. Fig. 4 is a comparison of the amino acid sequences of the LIV-1 polypeptides of SEQ ID NO:2 (see Green et al., *supra*) and SEQ ID NO:4 (disclosed herein). A six-amino acid insertion is observed at approximately amino acids 126 to 131 in the ECD, while

5 different amino acid sequences are observed at the C-terminal end of the polypeptides due to single nucleotide insertions (see amino acids beyond amino acid 736 of SEQ ID NO:2 and beyond amino acid 742 of SEQ ID NO:4).

Specifically, DNA 164647 isolation was performed according to the following procedure. Two adjacent 5'phosphorylated primers, LIV1-INV5' and LIV1-INV3', were designed on opposite strands. These primers, SEQ ID NO:6 and SEQ ID NO:7, were used in an inverse PCR reaction. In a 50ul reaction, the following reagents were

10 added: 50ng of a bone marrow cDNA library in a modified pRK5D vector, which was propagated in a methylation positive bacteria; 50 picomoles of each PCR primer; 10nmoles of each deoxynucleotide triphosphate, 5ul of Pfu 10x buffer (Stratagene, La Jolla CA), and 1ul of Pfu Turbo (Stratagene, La Jolla CA). The plasmid vector pRK5 (4,661 bp) has been described (EP 307,247 published 15 March 1989). The modified pRK5 vector (pRK5.tk.neo) is a derivative of pRK5 in which the neomycin resistance gene is inserted thereby allowing for selection of G418-

15 resistant clones.

The PCR cycle conditions were one cycle at 94°C for 3minutes, then 94°C for 30 seconds, 65°C for 30 seconds, 72°C for 13 minutes for 20 cycles. PCR conditions may, of course, be modified to meet specific needs of amplification. The PCR reaction generated a linear 5' phosphorylated amplicon that contained the LIV-1 cDNA insert plus the pRK5D vector.

20 Next, 10ul of the completed PCR reaction was ligated in a 100ul reaction containing the following other reagents: 10ul 10x T4 DNA ligase buffer (New England BioLabs, Beverly, MA), 4ul T4 DNA ligase (New England BioLabs, Beverly, MA), 76ul H_2O. The ligation was allowed to incubate at ambient temperature for 1 hour on the bench top.

After 1 hour of ligation, 2ul of the restriction enzyme DpnI (New England BioLabs, Beverly, MA) was

25 added to the ligation reaction and the digestion was allowed to continue for 1 hour at 37°C. DpnI will specifically digest methylated DNA and not unmethylated DNA; therefore, the original cDNA library which was used as a template will be digested, leaving only the LIV-1/vector amplicon intact. After the completion of the digestion the sample was cleaned using the QIAquick PCR purification kit (Qiagen, Valencia, CA), eluted in 30ul of elution buffer or H_2O, and then ethanol precipitated. The pellet was resuspended in 2ul of H_2O and the entire sample was

30 then used to transformed bacteria.

Transformation was done by electroporation into DH10B electromax competent bacteria (Life Technologies, Rockville, MD). The transformed bacteria were plated on Luria broth agar plates and colonies allowed to grow overnight at 37°C.

The next day, the colonies were lifted onto a nylon membrane, denatured, renatured and probed with a [32]P-

35 ATP kinase-labeled, LIV-1-specific probe (SEQ ID NO:8). DNA from positive colonies was sequenced to confirm that the sequence did not contain point mutations introduced by the PCR reaction.

LIV-1 Polypeptide Production: The description below relates primarily to production of LIV-1

polypeptides by culturing cells transformed or transfected with a vector containing LIV-1-encoding nucleic acid. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare LIV-1 polypeptides. For instance, the LIV-1 polypeptide sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques [see, *e.g.*, Stewart et al., Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, CA (1969); Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963)]. *In vitro* protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer's instructions. Various portions of the LIV-1 polypeptide may be chemically synthesized separately and combined using chemical or enzymatic methods to produce the full-length LIV-1.

i.   Alternative Methods for Synthesis or Isolation of LIV-1- encoding DNA .

Disclosed herein are various, non-limiting examples of methods for isolating or synthesizing DNA as well as expressing and producing proteins. These methods are applicable and useful for the production and isolation of LIV-1-164647 gene, mRNA, and protein, or fragments thereof.

DNA encoding LIV-1-164647 polypeptide, homologues, variants, or portions thereof, may be produced by direct DNA synthesis using standard nucleic acid synthetic techniques [see, *e.g.*, Gait, M.J., *Oligonucleotide Synthesis*, IRL Press, Oxford, 1984]. DNA synthesis *in vitro* may be performed using manual techniques or by automation. Automated oliogonucleotide synthesis may be accomplished, for instance, using standard techniques. Various portions of the LIV-1-encoding nucleic acid sequence may be chemically synthesized separately and combined using chemical or enzymatic methods to produce the full-length LIV-1-encoding sequence.

Alternatively, DNA encoding LIV-1 may be obtained from a cDNA library prepared from tissue believed to possess the LIV-1 mRNA and to express it at a detectable level. Accordingly, human LIV-1 DNA can be conveniently obtained from a cDNA library prepared from human tissue, such as described in the Examples. The LIV-1-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the LIV-1 polypeptide, or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding LIV-1 is to use PCR methodology [Sambrook et al., *supra*; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

The Examples below describe techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ^{32}P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., *supra*.

Sequences identified in such library screening methods can be compared and aligned to other known sequences deposited and available in public databases such as GenBank or other private sequence databases.

Sequence identity (at either the amino acid or nucleotide level) within defined regions of the molecule or across the full-length sequence can be determined through sequence alignment using computer software programs such as ALIGN, DNAstar, and INHERIT which employ various algorithms to measure homology.

Nucleic acid having protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

                  ii.   Selection and Transformation of Host Cells

Host cells are transfected or transformed with expression or cloning vectors described herein for LIV-1 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991) and Sambrook et al., supra.

Methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with Agrobacterium tumefaciens is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb. Virology, 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transformations have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al.. J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include, but are not limited to, eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as E. coli. Various E. coli strains are publicly available, such as E. coli K12 strain MM294 (ATCC 31,446); E. coli X1776 (ATCC 31,537); E. coli strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635).

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for LIV-1-encoding vectors. Saccharomyces cerevisiae is a commonly used lower eukaryotic host microorganism.

Suitable host cells for the expression of LIV-1 are derived from multicellular organisms. Examples of

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invertebrate cells include insect cells such as Drosophila S2 and Spodoptera Sf9. as well as plant cells. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture. Graham et al.. J. Gen Virol., 36:59 (1977)); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); human lung cells (W138, ATCC CCL75); human liver cells (Hep G2. HB 8065); and mouse mammary tumor (MMT 060562, ATCC CCL51). The selection of the appropriate host cell is deemed to be within the skill in the art.

iii. Selection and Use of a Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding LIV-1 may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector may. for example, be in the form of a plasmid, cosmid. viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general. DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include. but are not limited to, one or more of a signal sequence. an origin of replication, one or more marker genes. an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

The LIV-1 polypeptide may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general. the signal sequence may be a component of the vector. or it may be a part of the LIV-1-encoding DNA that is inserted into the vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase. penicillinase. lpp. or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be. e.g., the yeast invertase leader. alpha factor leader (including Saccharomyces and Kluyveromyces α-factor leaders, the latter described in U.S. Patent No. 5,010,182), or acid phosphatase leader. the C. albicans glucoamylase leader (EP 362.179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus. VSV or BPV) are useful for cloning vectors in mammalian cells.

Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins. e.g.. ampicillin. neomycin. methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g.. the gene encoding D-alanine racemase for Bacilli.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the LIV-1-encoding nucleic acid. such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity. prepared and propagated as described by Urlaub et al.. Proc. Natl. Acad. Sci. USA. 77:4216 (1980). A suitable selection gene

5   for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 [Stinchcomb et al.. Nature, 282:39 (1979); Kingsman et al.. Gene, 7:141 (1979): Tschemper et al.. Gene, 10:157 (1980)]. The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan. for example, ATCC No. 44076 or PEP4-1 [Jones. Genetics, 85:12 (1977)].

Expression and cloning vectors usually contain a promoter operably linked to the LIV-1-encoding nucleic

10   acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems [Chang et al., Nature. 275:615 (1978); Goeddel et al.. Nature, 281:544 (1979)]. alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel. Nucleic Acids Res.. 8:4057 (1980); EP 36.776]. and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. Promoters for use in bacterial systems also

15   will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding LIV-1.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)]. such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate

20   isomerase, 3-phosphoglycerate mutase. pyruvate kinase. triosephosphate isomerase. phosphoglucose isomerase. and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2. isocytochrome C. acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phos-

25   phate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73.657.

Transcription of LIV-1 from vectors in mammalian host cells is controlled. for example. by promoters obtained from the genomes of viruses such as polyoma virus. fowlpox virus (UK 2,211.504 published 5 July 1989). adenovirus (such as Adenovirus 2). bovine papilloma virus, avian sarcoma virus. cytomegalovirus. a retrovirus.

30   hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*. the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding a LIV-1 polypeptide by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA. usually about from 10 to 300 bp.

35   that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin. α-fetoprotein. and insulin). Typically, however. one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270),

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the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the LIV-1 coding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated

5   cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5'and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding LIV-1.

Still other methods, vectors, and host cells suitable for adaptation to the synthesis of LIV-1 in recombinant

10   vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

iv.   Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA,

15   77:5201-5205 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

20   Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence LIV-1 polypeptide or against a synthetic peptide based on the

25   DNA sequences provided herein or against exogenous sequence fused to LIV-1 DNA and encoding a specific antibody epitope.

v.   Production and Isolation of LIV-1 Polypeptide from Host Cells

Expression of LIV-1 in mammalian cells

This example illustrates preparation of a LIV-1 polypeptide by recombinant expression in

30   mammalian cells.

The vector, pRK5 (see EP 307,247, published March 15, 1989), is employed as the expression vector. Optionally, the LIV-1 DNA164647 is ligated into pRK5 with selected restriction enzymes to allow insertion of the LIV-1 DNA using ligation methods such as described in Sambrook et al., supra. The resulting vector is called pRK5-DNA164647.

35   In one embodiment, the selected host cells may be 293 cells. Human 293 cells (ATCC CCL 1573) are grown to confluence in tissue culture plates in medium such as DMEM supplemented with fetal calf serum and optionally, nutrient components and/or antibiotics. About 10 µg pRK5-DNA164647 DNA is mixed with about 1

μg DNA encoding the VA RNA gene [Thimmappaya et al.. Cell. 31:543 (1982)] and dissolved in 500 μl of 1 mM Tris-HCl. 0.1 mM EDTA. 0.227 M CaCl$_2$. To this mixture is added. dropwise. 500 μl of 50 mM HEPES (pH 7.35). 280 mM NaCl. 1.5 mM NaPO$_4$. and a precipitate is allowed to form for 10 minutes at 25°C. The precipitate is suspended and added to the 293 cells and allowed to settle for about four hours at 37°C. The culture medium is

5   aspirated off and 2 ml of 20% glycerol in PBS is added for 30 seconds. The 293 cells are then washed with serum free medium. fresh medium is added and the cells are incubated for about 1-5 days.

Approximately 24 hours after the transfections. the culture medium is removed and replaced with culture medium (alone) or culture medium containing 200 μCi/ml ^{35}S-cysteine and 200 μCi/ml ^{35}S-methionine. After a 12 hour incubation. the conditioned medium is collected. concentrated on a spin filter, and loaded onto a 15% SDS

10   gel. The processed gel may be dried and exposed to film for a selected period of time to reveal the presence of LIV-1 polypeptide. The cultures containing transfected cells may undergo further incubation (in serum free medium) and the medium is tested in selected bioassays.

In an alternative technique, LIV-1 DNA164647 may be introduced into 293 cells transiently using the dextran sulfate method described by Somparyrac et al.. Proc. Natl. Acad. Sci.. 12:7575 (1981). 293 cells are grown

15   to maximal density in a spinner flask and 700 μg pRK5-DNA164647 DNA is added. The cells are first concentrated from the spinner flask by centrifugation and washed with PBS. The DNA-dextran precipitate is incubated on the cell pellet for four hours. The cells are treated with 20% glycerol for 90 seconds. washed with tissue culture medium, and re-introduced into the spinner flask containing tissue culture medium, 5 μg/ml bovine insulin and 0.1 μg/ml bovine transferrin. After about four days. the conditioned media is centrifuged and filtered to remove cells

20   and debris. The sample containing expressed LIV-1 can then be concentrated and purified by any selected method. such as dialysis and/or column chromatography.

LIV-1 can be expressed in CHO cells. Following PCR amplification. the DNA164647 is subcloned in a CHO expression vector using standard techniques as described in Ausubel et al., *Current Protocols of Molecular Biology.* Unit 3.16, John Wiley and Sons (1997). CHO expression vectors are constructed to have compatible

25   restriction sites 5' and 3' of the DNA of interest to allow the convenient shuttling of cDNA's. The vector uses expression in CHO cells is as described in Lucas *et al., Nucl. Acids Res.* 24: 9 (1774-1779 (1996). and uses the SV40 early promoter/enhancer to drive expression of the cDNA of interest and dihydrofolate reductase (DHFR). DHFR expression permits selection for stable maintenance of the plasmid following transfection.

Approximately twelve micrograms of the desired plasmid DNA is introduced into approximately 10 million

30   CHO cells using commercially available transfection reagents, such as Superfect® (Quiagen). Dosper® or Fugene® (Boehringer Mannheim). The cells are grown and described in Lucas *et al.*. supra. Approximately 3 x 10^{-7} cells are frozen in an ampule for further growth and production as described below.

The ampules containing the plasmid DNA are thawed by placement into a water bath and mixed by vortexing. The contents are pipetted into a centrifuge tube containing 10 mLs of media and centrifuged at 1000 rpm

35   for 5 minutes. The supernatant is aspirated and the cells are resuspended in 10 mL of selective media (0.2 μm filtered PS20 with 5% 0.2 μm diafiltered fetal bovine serum). The cells are then aliquoted into a 100 mL spinner containing 90 mL of selective media. After 1-2 days, the cells are transferred into a 250 mL spinner filled with 150

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mL selective growth medium and incubated at 37°C. After another 2-3 days, a 250 mL 500 mL and 2000 mL

spinners are seeded with 3 x 10^5 cells/mL. The cell media is exchanged with fresh media by centrifugation and

resuspension in production medium. Although any suitable CHO media may be employed, a production medium

described in US Patent No. 5,122,469, issued June 16, 1992 was actually used. 3L production spinner is seeded

5   at 1.2 x 10^6 cells/mL. On day 0, the cell number pH are determined. On day 1, the spinner is sampled and sparging

with filtered air is commenced. On day 2, the spinner is sampled, the temperature shifted to 33°C. and 30 mL of

500 g/L glucose and 0.6 mL of 10% antifoam (e.g., 35% polydimethylsiloxane emulsion, Dow Corning 365 Medical

Grade Emulsion). Throughout the production, pH is adjusted as necessary to maintain a pH of about 7.2. After 10

days, or until viability dropped below 70%, the cell culture is harvested by centrifugtion and filtering through a

10   0.22 μm filter. The filtrate is either stored at 4°C or immediately loaded onto columns for purification.

For the poly-His tagged constructs, the proteins are purified using a Ni-NTA column (Qiagen). Before

purification, imidazole is added to the conditioned media to a concentration of 5 mM. The conditioned media is

pumped onto a 6 ml Ni-NTA column equilibrated in 20 mM Hepes, pH 7.4, buffer containing 0.3 M NaCl and 5

mM imidazole at a flow rate of 4-5 ml/min. at 4°C. After loading, the column is washed with additional

15   equilibration buffer and the protein eluted with equilibration buffer containing 0.25 M imidazole. The highly

purified protein is subsequently desalted into a storage buffer containing 10 mM Hepes. 0.14 M NaCl and 4%

mannitol, pH 6.8, with a 25 ml G25 Superfine (Pharmacia) column and stored at -80°C.

LIV-1 may be produced by transient or stable expression in a host cell, such as COS cells, using standard

techniques.

20   Expression of LIV-1 in Yeast

The following method describes recombinant expression of LIV-1 in yeast.

First, yeast expression vectors are constructed for intracellular production or secretion of LIV-1 from the

ADH2/GAPDH promoter. DNA164647 encoding a LIV-1 and the promoter is inserted into suitable restriction

enzyme sites in the selected plasmid to direct intracellular expression of LIV-1. For secretion, DNA encoding LIV-1

25   can be cloned into the selected plasmid, together with DNA encoding the ADH2/GAPDH promoter, a signal

peptide, such as a mammalian signal peptide, or, for example, a yeast alpha-factor or invertase secretory

signal/leader sequence, and linker sequences (if needed) for expression of LIV-1.

Yeast cells, such as yeast strain AB110, can then be transformed with the expression plasmids described

above and cultured in selected fermentation media. The transformed yeast supernatants can be analyzed by

30   precipitation with 10% trichloroacetic acid and separation by SDS-PAGE, followed by staining of the gels with

Coomassie Blue stain.

Recombinant LIV-1 can subsequently be isolated and purified by removing the yeast cells from the

fermentation medium by centrifugation and then concentrating the medium using selected cartridge filters. The

concentrate containing LIV-1 may further be purified using selected column chromatography resins.

Expression of LIV-1 in Baculovirus-Infected Insect Cells

The following method describes recombinant LIV-1 expression in Baculovirus-infected insect cells.

The sequence coding for LIV-1 is fused upstream of an epitope tag contained within a baculovirus expression vector. Such epitope tags include poly-His tags and immunoglobulin tags (like Fc regions of IgG). A variety of plasmids may be employed, including plasmids derived from commercially available plasmids such as pVL1393 (Novagen). Briefly, a nucleic acid sequence encoding LIV-1 or the desired portion of the coding sequence of LIV-1 (such as the sequence encoding the extracellular domain of a transmembrane protein or the sequence encoding the mature protein if the protein is extracellular) is amplified by PCR with primers complementary to the 5' and 3' regions. The 5' primer may incorporate flanking (selected) restriction enzyme sites. The product is then digested with those selected restriction enzymes and subcloned into the expression vector.

Recombinant baculovirus is generated by co-transfecting the above plasmid and BaculoGold™ virus DNA (Pharmingen) into Spodoptera frugiperda ("Sf9") cells (ATCC CRL 1711) using lipofectin (commercially available from GIBCO-BRL). After 4 - 5 days of incubation at 28°C, the released viruses are harvested and used for further amplifications. Viral infection and protein expression are performed as described by O'Reilley et al., Baculovirus expression vectors: A Laboratory Manual. Oxford: Oxford University Press (1994).

Expressed poly-His tagged LIV-1 can then be purified, for example, by Ni^{2+}-chelate affinity chromatography as follows. Extracts are prepared from recombinant virus-infected Sf9 cells as described by Rupert et al., Nature, 362:175-179 (1993). Briefly, Sf9 cells are washed, resuspended in sonication buffer (25 mL Hepes, pH 7.9; 12.5 mM $MgCl_2$; 0.1 mM EDTA; 10% glycerol; 0.1% NP-40; 0.4 M KCl), and sonicated twice for 20 seconds on ice. The sonicates are cleared by centrifugation, and the supernatant is diluted 50-fold in loading buffer (50 mM phosphate, 300 mM NaCl, 10% glycerol, pH 7.8) and filtered through a 0.45 μm filter. A Ni^{2+}-NTA agarose column (commercially available from Qiagen) is prepared with a bed volume of 5 mL, washed with 25 mL of water and equilibrated with 25 mL of loading buffer. The filtered cell extract is loaded onto the column 0.5 mL per minute. The column is washed to baseline A_{280} with loading buffer, at which point fraction collection is started. Next, the column is washed with a secondary wash buffer (50 mM phosphate; 300 mM NaCl, 10% glycerol, pH 6.0), which elutes non specifically bound portein. After reaching A_{280} baseline again, the column is developed with a 0 to 500 mM imidazole gradient in the secondary wash buffer. One mL fractions are collected and analyzed by SDS-PAGE and silver staining or Western blot with Ni^{2+}-NTA-conjugated to alkaline phosphatase (Qiagen). Fractions containing the eluted His_{10}-tagged LIV-1 are pooled and dialyzed against loading buffer. Alternatively, purification of the IgG tagged (or Fc tagged) LIV-1 can be performed using known chromatography techniques, including for instance, Protein A or Protein G column chromatography.

While the LIV-1 expression is performed in a 0.5-2L scale, it can be readily scaled up for larger (e.g. 8L) preparations. LIV-1 is also expressed as an IgG construct (immunoadhesin), in which the protein extracellular region is fused to an IgG1 constant region sequence containing the hinge, C_H2 and C_H3 domains and/or in poly-His tagged forms.

Following PCR amplification, the coding sequence is subcloned into a baculovirus expression vector

(pb.PH.IgG for IgG fusions and pb.PH.His.c for poly-His tagged proteins), and the vector and Baculogold®
baculovirus DNA (Pharmingen) is co-transfected into 105 *Spondoptera frugiperda* ("Sf9") cells (ATCC CRL 1711).
using Lipofectin (Gibco BRL). pb.PH.IgG and pb.PH.His are modifications of the commercially available
baculovirus expression vector pVL1393 (Pharmingen), with modified polylinker regions to include the His or Fc

5   tag sequences. The cells are grown in Hink's TNM-FH medium supplemented with 10% FBS (Hyclone). Cells
are incubated for 5 days at 28°C. The supernatant is harvested and subsequently used for the first viral amplification
by infecting Sf9 cells in Hink's TNM-FH medium supplemented with 10% FBS at an approximate multiplicity of
infection (MOI) of 10. Cells are incubated for 3 days at 28°C. The supernatant is harvested and the expression of
the constructs in the baculovirus expression vector is determined by batch binding of 1 mL of supernatant to 25 mL

10   of of Ni-NTA beads (Qiagen) for histidine tagged proteins or Protein-A Sepharose CL-4B beads (Pharmacia) for
IgG tagged proteins followed by SDS-PAGE analysis comparing to a known concentration of protein standard by
Coomassie blue staining.

The first viral amplification supernatant is used to infect a spinner culture (500 mL) of Sf9 cells grown in
ESF-921 medium (Expression Systems LLC) at an approximate MOI of 0.1. Cells are incubated for 3 days at 28°C.

15   The supernatant is harvested and filtered. Batch binding and SDS-PAGE analysis is repeated, as necessary, until
expression of the spinner culture is confirmed.

The conditioned medium from the transfected cells (0.5 to 3 L) is harvested by centrifugation to remove
the cells and filtered through 0.22 micron filters. For the poly-His taged constructs, the protein construct are
purified using a Ni-NTA column (Qiagen). Before purification, imidazole is added to the conditioned media to a

20   concentration of 5 mM. The conditioned media are pumped onto a 6 mL Ni-NTA column equilibrated in 20 mM
Hepes, pH 7.4, buffer containing 0.3 M NaCl and 5 mM imidazole at a flow rate of 4-5 mL/min. at 4°C. After
loading, the column is washed with additional equilibration buffer and the protein eluted with equilibration buffer
containing 0.25 M imidazole. The highly purified protein is subsequently desalted into a storage buffer
containing 10 mM Hepes. 0.14 M NaCl and 4% mannitol, pH 6.8. with a 25 mL G25 Superfine (Pharmacia) column

25   and stored at -80°C.

Immunoadhesin (Fc containing) constructs of proteins are purified from the conditioned medium as
follows. The conditioned medium is pumped onto a 5 mL Protein A column (Pharmacia) which had been
equilibrated in 20 mM sodium phosphate buffer, pH 6.8. After loading, the column is washed extensively with
equilibration buffer before elution with 100 mM citric acid, pH 3.5. The eluted protein is immediately neutralized

30   by collecting 1 mL fractions into tubes containing 275 of 1 M Tris buffer, pH 9. The highly purified protein is
subsequently desalted into storage buffer as described above for the poly-His tagged proteins. The homogeneity
of the proteins is verified by SDS-PAGE and N-terminal amino acid sequencing by Edman degradation.

                              vi.   Purification of Polypeptide

Forms of LIV-1 polypeptides may be recovered from culture medium or from host cell lysates.

35   If membrane-bound, it can be released from the membrane using a suitable detergent solution (*e.g.* Triton-X 100)
or by enzymatic cleavage. Cells employed in expression of LIV-1 can be disrupted by various physical or chemical
means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

It may be desired to purify LIV-1 from recombinant cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; 5 protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of the LIV-1 polypeptides. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, Methods in Enzymology, 182 (1990); Scopes. Protein Purification: Principles and Practice, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular LIV-1 polypeptide 10 produced.

Example 3.   Preparation and Efficacy of Anti-LIV-1 Antibody

A description follows as to exemplary techniques for the production of the anti-LIV-1 antibodies used in accordance with the present invention. Techniques for the production of anti-ErbB2 antibodies may be found in the ErbB2 publications listed *supra* and incorporated herein by reference.

15 The LIV-1 antigen particularly useful for production of antibodies may be, *e.g.*, a soluble form of the extracellular domain of LIV-1-164647 or a portion thereof, containing an anitgenic epitope. The extracellular domain region is indicated in Fig. 1. Alternatively, cells expressing LIV-1 at their cell surface can be used to generate antibodies (*e.g.* NIH-3T3 cells transformed to overexpress LIV-1). Other forms of LIV-1 useful for generating antibodies will be apparent to those skilled in the art.

20   *(i)   Polyclonal antibodies*

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, 25 maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N=C=NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.*, 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete 30 adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in 35 recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii)   Monoclonal antibodies

59

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies. *i.e..* the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

5   For example. the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4.816.567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster. is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro.* Lymphocytes

10   then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice,* pp.59-103 [Academic Press, 1986]).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT

15   or HPRT), the culture medium for the hybridomas typically will include hypoxanthine. aminopterin. and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse

20   tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, 10801 University Boulevard. Manassas, VA 20110-2209. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications,* pp. 51-63 [Marcel Dekker, Inc., New York, 1987]).

25   Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably. the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay. such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example. be determined by the Scatchard analysis

30   of Munson *et al., Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity. affinity. and/or activity. the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding. *Monoclonal Antibodies: Principles and Practice,* pp.59-103 [Academic Press, 1986]). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown

35   *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium. ascites fluid, or serum by conventional immunoglobulin purification procedures such as. for example. protein A-

Sepharose. hydroxylapatite chromatography, gel electrophoresis, dialysis. or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g.. by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors. which are then transfected into host cells such as E. coli cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al.. Curr. Opinion in Immunol., 5:256-262 (1993) and Plückthun. Immunol. Revs., 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990). Clackson et al.. Nature, 352:624-628 (1991) and Marks et al.. J. Mol. Biol.. 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively. using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al.. Bio/Technology. 10:779-783 [1992]). as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al.. Nuc. Acids. Res.. 21:2265-2266 [1993]). Thus. these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4.816,567: Morrison, et al., Proc. Natl Acad. Sci. USA. 81:6851 [1984]), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

More specifically, an anti-LIV-1 monoclonal antibody is prepared as follows. An anti-LIV-1 $IgG_1\kappa$ murine monoclonal antibody, preferably specific for the extracellular domain of LIV-1-164647 protein. is produced using procedures like those described in Fendly et al.. Cancer Research 5o:1550-1558(1990) and WO89/06692. coupled with ordinary skill in the art. Briefly, LIV-1-expressing cells (preferably cells expressing LIV-1 encoded by DNA164647) are harvested with phosphate buffered saline (PBS) containing 25mM EDTA and used to immunize BALB/c mice. The mice are given injections i.p. of 10^7 cells in 0.5 ml PBS on weeks, 0. 2. 5 and 7, for example. The mice with antisera that immunoprecipitates ^{32}P-labeled LIV-1 protein. preferably by binding to the extracellular domain or extracellular fragments, are given i.p. injections of a wheat germ agglutinin-Sepharose (WGA) purified LIV-1 membrane extract on weeks 9 and 13. This is followed by an i.v. injection of 0.1 ml of the LIV-1 preparation and the splenocytes are fused with mouse myeloma line X63-Ag8.653. for example. Hybridoma supernatants are screened for LIV-1-binding by ELISA and radioimmunoprecipitation. MOPC-21 (IgG1), (Cappell, Durham, NC). is used as an isotype-matched control. The disclosed method of preparing an anti-LIV-1

antibody is provided as an example since other methods for producing monoclonal antibodies are contemplated.

The treatment is performed with a humanized version of the murine anti-LIV-1 antibody. The humanized antibody is engineered by inserting the complementarity determining regions of the murine anti-LIV-1 antibody into the framework of a consensus human immunoglobulin IgG$_1$ (IgG$_1$) (see for example the process used in Carter *et*
5   *al.. Proc. Natl. Acad. Sci. USA* 89:4285-4289 [1992]). The resulting humanized anti-LIV-1 monoclonal antibody preferably has high affinity for the extracellular domain of LIV-1 protein and inhibits, *in vitro* and *in vivo* and in human xenografts, the growth of breast cancer cells, lung cancer cells. prostate cancer cells or other cell that overexpresses LIV-1 protein. Preferably the anti-LIV-1 antibodies of the invention inhibit tumor cell growth greater than 20%, most preferably greater than 50%. *in vitro*. The preferred anti-LIV-1 monoclonal antibody of the
10 invention is also clinically active, as a single agent or in combination with a cytotoxic or other cell growth-inhibiting agent, in patients with LIV-1-overexpressing metastatic breast cancers. or lung, prostate or other cancers. Anti-LIV-1 monoclonal antibody is produced by a genetically engineered Chinese Hamster Ovary (CHO) cell line, grown in large scale, that secretes the antibody into the culture medium. The antibody is purified from the CHO culture media using standard chromatographic and filtration methods. Each lot of antibody is assayed to verify identity, purity.
15 and potency, as well as to meet Food and Drug Administration requirements for sterility and safety.

When used to kill human cancer cells *in vitro* for diagnostic purposes or to test the potency of a lot of antibodies, the antibodies will typically be added to a culture of LIV-1-overexpressing cells. particularly cancerous cells, that do not also overexpress ErbB2. As a control, the antibodies will also be added to a culture of cells that do not overexpress LIV-1. The antibodies are added to the cell culture medium at a concentration of at least
20 approximately 10 nM. The formulation and mode of administration for *in vitro* use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques to determine the presence or degree of cancer.

Cytotoxic radiopharmaceuticals for treating cancer may be made by conjugating radioactive isotopes (*e.g.* I, Y, Pr) to the antibodies. The term "cytotoxic moiety" as used herein is intended to include such isotopes.
25   In another embodiment, liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding a growth factor receptor. Since there are many receptro sites. this method permits delivery of large amounts of drug to the correct cell type.

Antibody dependent cellular cytotoxicity (ADCC) is contemplated as a method of targeting cytotoxic effects to cancerous cells overexpressing LIV-1 protein. The present invention involves a method based on the use
30 of antibodies withic are (a) directed against the extracellular domain of LIV-1 protein, and (b) belong to a subclass or isotype that is capable of mediating the lysis of tumor cells to which the antibody molecule binds. More specifically, these antibodies should belong to a subclass or isotype that, upon complexing with growth factor receptors, activates serum complement and/or mediates antibody dependent cellular cytotoxicity (ADCC) by activating effector cells such as natural killer cells or macrophages.
35   The present invention is also directed to the general use of these antibodies, in their native form, for therapy of human tumors that overexpress the LIV-1 protein. For example. many IgG2a and IgG3 mouse antibodies which bind tumor-associated cell surface antigens can be used in vivo for tumor therapy. In fact. since LIV-1 is

present on a variety of tumors, the subject antibodies and their therapeutic use have general applicability.

<center>(iii)   Humanized and human antibodies</center>

Methods for humanizing non-human antibodies are well known in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human.

5   These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 [1988]), by substituting rodent hypervariable regions (e.g., CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric

10   antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized

15   antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Sims et al., J. Immunol., 151:2296 (1993); Chothia et al., J. Mol. Biol., 196:901 [1987]). Another method uses a particular framework region derived from the consensus sequence of all

20   human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immnol., 151:2623 [1993]).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are

25   prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate

30   immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon

35   immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody

<center>63</center>

production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA. 90:2551 (1993); Jakobovits et al., Nature. 362:255-258 (1993): Bruggermann et al., Year in Immuno.. 7:33 (1993). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al.. J. Mol. Biol., 227:381 (1991); Marks et al.. J. Mol. Biol.. 222:581-597 [1991]).

(iv)   Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g.. Morimoto et al. . Journal of Biochemical and Biophysical Methods 24:107-117 (1992) and Brennan et al., Science. 229:81 [1985]). However, these fragments can now be produced directly by recombinant host cells. For example. the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from E. coli and chemically coupled to form F(ab')$_2$ fragments (Carter et al.. Bio/Technology 10:163-167 [1992]). According to another approach, F(ab')$_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185.

(v)   Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the LIV-1 protein. For example, one arm may bind a first epitope in the extracellular domain of LIV-1 protein, while the other may bind a different LIV-1 epitope. Alternatively, an anti-LIV-1 arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T-cell receptor molecule (e.g. CD2 or CD3). or Fc receptors for IgG (FcγR). such as FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) so as to focus cellular defense mechanisms to the ErbB2-expressing cell. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express LIV-1 by binding to the extracellular domain of the LIV-1 gene product. These antibodies possess a LIV-1 extracellular domain-binding arm and an arm which binds the cytotoxic agent (e.g. saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab')$_2$ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs. where the two chains have different specificities (Millstein et al., Nature, 305:537-539 [1983]). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al.. EMBO J., 10:3655-3659 (1991).

According to a different approach. antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge. CH2. and CH3

regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding. present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and. if desired. the immunoglobulin light chain. are inserted into separate expression vectors. and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is. however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach. the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations. as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh et al.. Methods in Enzymology, 121:210 (1986).

According to another approach described in WO96/27011. the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example. one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4.676.980). and for treatment of HIV infection (WO 91/00360. WO 92/200373. and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art. and are disclosed in US Patent No. 4,676,980. along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al.. Science, 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')$_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective

immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al.*, *J. Exp. Med.*, 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody F(ab')$_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets. Similar techniques are used to prepare a bispecific antibody able to bind cells overexpressing LIV-1 but not overpression ErbB2.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al.*, *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V$_H$) connected to a light-chain variable domain (V$_L$) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V$_H$ and V$_L$ domains of one fragment are forced to pair with the complementary V$_L$ and V$_H$ domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, *J. Immunol.*, 152:5368 (1994).

Antibodies with more than two valencies are also contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60 (1991).

(vi)   *Screening for antibodies with the desired properties*

Techniques for generating antibodies have been described above. Those antibodies having the characteristics described herein are selected.

To select for antibodies which induce cell death, loss of membrane integrity as indicated by, *e.g.*, PI, trypan blue or 7AAD uptake is assessed relative to control. The preferred assay is the "PI uptake assay using BT474 cells". According to this assay, BT474 cells (which can be obtained from the American Type Culture Collection [Manassas, VA]) are cultured in Dulbecco's Modified Eagle Medium (D-MEM):Ham's F-12 (50:50) supplemented with 10% heat-inactivated FBS (Hyclone) and 2 mM L-glutamine. (Thus, the assay is performed in the absence of complement and immune effector cells). The BT474 cells are seeded at a density of 3 x 10^6 per dish in 100 x 20 mm dishes and allowed to attach overnight. The medium is then removed and replaced with fresh medium alone or medium containing 10μg/ml of the appropriate MAb. The cells are incubated for a 3 day time period. Following each treatment, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged at 1200rpm for 5 minutes at 4°C, the pellet resuspended in 3 ml ice cold Ca^{2+} binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl$_2$) and aliquoted into 35 mm strainer-capped 12 x 75 tubes (1 ml per tube, 3 tubes per

treatment group) for removal of cell clumps. Tubes then receive PI (10μg/ml). Samples may be analyzed using a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of cell death as determined by PI uptake are selected.

5   In order to select for antibodies which induce apoptosis, an "annexin binding assay using BT474 cells" is available. The BT474 cells are cultured and seeded in dishes as discussed in the preceding paragraph. The medium is then removed and replaced with fresh medium alone or medium containing 10μg/ml of the MAb. Following a three day incubation period, monolayers are washed with PBS and detached by trypsinization. Cells are then centrifuged, resuspended in Ca^{2+} binding buffer and aliquoted into tubes as discussed above for the cell death assay. Tubes then receive labeled annexin (e.g. annexin V-FTIC) (1μg/ml). Samples may be analyzed using

10   a FACSCAN™ flow cytometer and FACSCONVERT™ CellQuest software (Becton Dickinson). Those antibodies which induce statistically significant levels of annexin binding relative to control are selected as apoptosis-inducing antibodies.

In addition to the annexin binding assay, a "DNA staining assay using BT474 cells" is available. In order to perform this assay, BT474 cells which have been treated with the antibody of interest as described in the

15   preceding two paragraphs are incubated with 9μg/ml HOECHST 33342™ for 2 hr at $37^{0}C$, then analyzed on an EPICS ELITE™ flow cytometer (Coulter Corporation) using MODFIT LT™ software (Verity Software House). Antibodies which induce a change in the percentage of apoptotic cells which is 2 fold or greater (and preferably 3 fold or greater) than untreated cells (up to 100% apoptotic cells) may be selected as pro-apoptotic antibodies using this assay.

20   To screen for antibodies that bind to an epitope on the extracellular domain of LIV-1-164647, a routine cross-blocking assay such as that described in *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed. Alternatively, epitope mapping can be performed by methods known in the art (see, for example, methods used for the epitope-mapping of the extracellular domain of ErbB2 as determined by truncation mutant analysis and site-directed mutagenesis (Nakamura *et al. J. of Virology* 67(10):6179-

25   6191 [Oct 1993]; Renz *et al. J. Cell Biol.* 125(6):1395-1406 [Jun 1994]). In addition, where a particular amino acid sequence is suspected of forming all or a substantial portion of an epitope, a polypeptide consisting of that sequence may be contacted with the antibody and tested, using standard techniques. for its ability to compete for binding to the LIV-1-164647, such as the LIV-1-164647 ECD.

To identify anti-LIV-1 antibodies which inhibit growth of LIV-1-expressing cells in cell culture by 50-

30   100%, an assay can be performed generally as follows: LIV-1-expressing cells are grown in a 1:1 mixture of F12 and DMEM medium supplemented with 10% fetal bovine serum, glutamine and penicillinstreptomycin. The LIV-1-expressing cells are plated at 20,000 cells in a 35mm cell culture dish (2 mls/35 mm dish). 2.5μg/ml of the anti-LIV-1 antibody is added per dish. After six days, the number of cells, compared to untreated cells are counted using an electronic COULTER™ cell counter. Those antibodies which inhibit growth of the LIV-1-expressing cells by 50-

35   100% are selected for combination with the apoptotic antibodies as desired.

(vii)   Effector function engineering

It may be desirable to modify the antibody of the invention with respect to effector function, so

as to enhance the effectiveness of the antibody in treating cancer, for example. For example, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al., J. Exp Med.* 176:1191-

5   1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al. Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:219-230 (1989).

10   *(viii)   Immunoconjugates*

The invention also pertains to immunoconjugates comprising the antibody described herein conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (*e.g.* an enzymatically active toxin of bacterial, fungal, plant or animal origin. or fragments thereof), or a radioactive isotope (*i.e.*, a radioconjugate).

Chemotherapeutic agents useful in the generation of such immunoconjugates have been described above.

15   Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain. nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for

20   the production of radioconjugated anti-ErbB2 antibodies. Examples include ^{212}Bi, ^{131}I, ^{131}In, ^{90}Y and ^{186}Re.

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine). bis-diazonium derivatives

25   (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate). and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al. Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody. See WO94/11026.

30   In another embodiment, the antibody may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" (*e.g.* avidin) which is conjugated to a cytotoxic agent (*e.g.* a radionucleotide).

                                   (ix)   Immunoliposomes

35   The anti-LIV-1 antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos.

4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab'

5   fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin *et al.* *J. Biol. Chem.* 257: 286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon *et al. J. National Cancer Inst.*81(19)1484 (1989).

                    (x)   Antibody Dependent Enzyme Mediated Prodrug Therapy (ADEPT)

The antibodies of the present invention may also be used in ADEPT by conjugating the antibody to a

10   prodrug-activating enzyme which converts a prodrug (*e.g.* a peptidyl chemotherapeutic agent, see WO81/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of the immunoconjugate useful for ADEPT includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase

15   useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic 5-fluorocytosine into the anti-cancer drug, 5-fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate-

20   cleaving enzymes such as β-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; β-lactamase useful for converting drugs derivatized with β-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active

25   drugs (see, *e.g.*, Massey, *Nature* 328: 457-458 [1987]). Antibody-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

The enzymes of this invention can be covalently bound to the anti-LIV-1 antibodies by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antibody of the invention linked to at least a

30   functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.*, Neuberger *et al.*, *Nature*, 312: 604-608 [1984]).

                    (xi)   Antibody-salvage receptor binding epitope fusions

In certain embodiments of the invention, it may be desirable to use an antibody fragment, rather than an intact antibody, to increase tumor penetration, for example. In this case, it may be desirable to modify the

35   antibody fragment in order to increase its serum half life. This may be achieved, for example, by incorporation of a salvage receptor binding epitope into the antibody fragment (*e.g.* by mutation of the appropriate region in the antibody fragment or by incorporating the epitope into a peptide tag that is then fused to the antibody fragment at

either end or in the middle. *e.g..* by DNA or peptide synthesis).

A systematic method for preparing such an antibody variant having an increased *in vivo* half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified. the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer *in vivo* half-life than that of the original antibody. If the antibody variant does not have a longer *in vivo* half-life upon testing. its sequence is further altered to include the sequence and conformation of the identified binding epitope. The altered antibody is tested for longer *in vivo* half-life, and this process is continued until a molecule is obtained that exhibits a longer *in vivo* half-life.

The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above. and its nature will depend, *e.g.*, on the type of antibody being modified. The transfer is made such that the antibody of interest still possesses the biological activities described herein.

The epitope preferably constitutes a region wherein any one or more amino acid residues from one or two loops of a Fc domain are transferred to an analogous position of the antibody fragment. Even more preferably. three or more residues from one or two loops of the Fc domain are transferred. Still more preferred. the epitope is taken from the C_H2 domain of the Fc region (*e.g.*. of an IgG) and transferred to the C_H1, C_H3. or V_H region, or more than one such region, of the antibody. Alternatively. the epitope is taken from the C_H2 domain of the Fc region and transferred to the C_L region or V_L region. or both, of the antibody fragment.

(xii)   Purification of anti-LIV-1 antibody

When using recombinant techniques. the antibody can be produced intracellularly. in the periplasmic space, or directly secreted into the medium. If the antibody is produced intracellularly. as a first step. the particulate debris, either host cells or lysed fragments, is removed, for example. by centrifugation or ultrafiltration. Carter *et al.. Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli.* Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5). EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min. Cell debris can be removed by centrifugation. Where the antibody is secreted into the medium. supernatants from such expression systems are preferably first concentrated using a commercially available protein concentration filter. for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

The antibody composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis. and affinity chromatography. with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc domain that is present in the antibody. Protein A can be used to purify antibodies that are based on human $\gamma1$, $\gamma2$. or $\gamma4$ heavy chains (Lindmark *et al.. J. Immunol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human $\gamma3$ (Guss *et al.. EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter

processing times than can be achieved with agarose. Where the antibody comprises a C_H3 domain, the Bakerbond ABX™ resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSE™ chromatography on an anion or cation exchange resin (such as a

5   polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the antibody to be recovered.

Following any preliminary purification step(s), the mixture comprising the antibody of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at low salt concentrations (*e.g.* from about 0-0.25M salt).

10   **Example 4.   LIV-1 is expressed on the cell surface**

Analysis of the deduced amino acid sequence of LIV-1 indicated that a portion of the protein exists as an extracellular domain (ECD). To verify this finding, the ECD was expressed, purified, and used as an antigen for the development of anti-LIV-1 ECD antibodies. The antibodies were contacted with cells expressing the full length LIV-1 protein. This Example shows that anti-LIV-1 ECD antibodies bound to LIV-1-expressing cells,

15   demonstrating that LIV-1 comprises an extracellular domain.

Expression of LIV-1 ECD in *E. coli*

To obtain samples of the LIV-1 ECD to which antibodies could be raised, a polypeptide comprising the extracellular domain of LIV-1 was expressed in *E. coli* after first constructing a nucleic acid vector encoding the LIV-1 extracellular domain operably linked to an amino acid leader sequence. The following

20   procedures were used to prepare the nucleic acid construct and express the encoded protein.

DNA coding for amino acids 1-298, encoding the extracellular domain of mature LIV-1, was prepared by standard PCR techniques from a full length cDNA clone using the primers 5'-CAACATCAAATGCATCAACTTCATGAACTAAAAGCAGCTGCT-3' (SEQ ID NO:9) and 5'-GAGCTCGAGCGGCCGCTTAGGTCTTTGGAGGGATTTCAGCCTT-3' (SEQ ID NO:10). The PCR reaction

25   was divided in two: one half was digested with *Nsi*I and *Sac*I, while the other half was digested with *Sac*I and *Not*I. The *Nsi*I-*Sac*I DNA fragment encoding amino acids 1-166 and the *Sac*I-*Not*I fragment encoding amino acids 167-298 were isolated and ligated into the previously digested expression vector pST239, a pBR322-derived vector containing an N-terminal polyhis leader at the 3' end of which is an Nsil restriction site. The resulting LIV-1 ECD expression plasmid was designated pEl64647.

30   Transcriptional and translational control of expression in the ST239 vector were provided by the following genetic features. Transcriptional initiation was controlled by the *E. coli* alkaline phosphatase promoter (Kikuchi Y. at al., Nucleic Acids Res. 9:5671-5678 (1981)). The *trp* operon ribosome binding site was used for translation initiation (Yanofsky C. et al., Nucleic Acids Res. 9:6647-6668 (1981)). Translational termination was effected by the translation termination codon and the downstream λto transcriptional terminator (Scholtissek S. et al., Nucleic

35   Acids Res. 15:3185 (1987)), followed by the rare codon tRNA genes *pro2, argU,* and *glyT* (Komine Y., et al., J. Mol. Biol. 212:579-598(1990), Fournier M.J., et al., Microbiol. Rev. 49:379-397 (1985)).

To facilitate expression. the extracellular domain of LIV-1 was expressed in *E. coli* cytoplasm with a N-terminal poly-histidine leader sequence encoded within the ST239 expression vector. The amino acid sequence of this leader was: MKHQHQHQHQHQHQMHQ (SEQ ID NO:11). This leader sequence offered several advantages. First. translation initiation was optimized. In addition, purification was simplified by adsorption on a nickel

5   chelation column. Finally. the leader sequence was easily and efficiently removed, as desired. using the TAGZyme™ system (Unizyme Laboratories. Horsholn, Denmark).

Following construction of the expression plasmid, pE164647, and DNA sequence verification. the LIV-1 expression plasmid was transformed into the *E. coli* strain 58F3 (fhuAΔ(tonAΔ) lonΔ galE rpoHts(htpRts) ΔclpP lacIq ΔompTΔ (nmpc-fepE) ΔslyD). Transformants were initially cultured in Luria broth at 30 °C overnight. and

10   then diluted 100-fold into a phosphate-limiting media to induce the alkaline phosphatase promoter. After 24 hours at 30 °C with shaking, the cultures were centrifuged. and the cell pastes frozen until the start of purification.

Purification of LIV-1 ECD

A 0.5 liter fermentation of *E. coli* transformants expressing LIV-1 yielded approximately 6 - 10 grams of cell paste. The paste was resuspended in 10 volumes (w/v) of 7 M guanidine. 20 mM Tris, pH 8 buffer. Solid

15   sodium sulfite and sodium tetrathionate were added to make final concentrations of 0.1 M and 0.02 M. respectively. and the solution was stirred overnight at 4 °C. This sulfitolysis step resulted in a denatured protein having all cysteine residues blocked. The solution was centrifuged at 40 K rpm in a Beckman ultracentifuge for 30 min. The supernatant was diluted with 3-5 volumes of metal chelate column buffer (6 M guanidine, 20 mM Tris. pH 7.4) and filtered through 0.22 micron filters to clarify. A volume corresponding to 50 mls of the clarified extract was loaded

20   onto a 5 ml Qiagen Ni-NTA (nickel-nitrilotriacetic acid) metal chelate affinity column equilibrated in the metal chelate column buffer (QIAexpress® Protein Purification System, Qiagen, Valencia. CA USA). The column was washed with additional buffer containing 50 mM imidazole (Calbiochem, Utrol grade), pH 7.4. The protein was eluted with buffer containing 250 mM imidazole. Fractions containing the desired protein were pooled and stored at 4 °C. Protein concentration was estimated by its absorbance at 280 nm using the calculated extinction

25   coefficient based on its amino acid sequence. Protein for antibody production was obtained by reducing an aliquot of the Ni-NTA pool with dithiothreitol (50 mM final concentration) followed by extensive dialysis against 1 mM HCl.

Development of Monoclonal Antibodies to LIV-1 Protein

Monoclonal antibodies specific for the extracellular domain of LIV-1 were developed according to the

30   following procedures.

Ten Balb/c mice (Charles River Laboratories, Wilmington, DE) were hyperimmunized with recombinant polyhistidine-tagged human LIV-1 isolated from pE164647-transformed *E. coli* as described herein. The tagged LIV-1 protein in Ribi adjuvant (Ribi Immunochem Research. Inc.. Hamilton. MO) was administered to the mice. B-cells from five mice demonstrating high anti-LIV-1 antibody titers were fused with mouse myeloma cells

35   (X63.Ag8.653; American Type Culture Collection, Rockville. MD) using a modified protocol analogous to one

previously described (Kohler. G. and Milstein, C.. Nature 256: 495-497 (1975): Hongo. J.S. et al., Hybridoma 14:253-260 (1995)).

After 7-14 days. the supernatants were harvested and screened for antibody production and LIV-1 binding specificity by standard techniques using direct enzyme-linked immunosorbent assay (ELISA). Sixteen positive
5   clones, showing the highest immunobinding affinity after the second round of subcloning by limiting dilution. were injected into Pristane-primed mice (Freund, Y.R. and Blair. P.B.. J. Immunol. 129:2826-2830 (1982)) for *in vivo* production of monoclonal antibody. The ascites fluids from these mice were pooled and purified by Protein A affinity chromatography (Pharmacia fast protein liquid chromatography (FPLC) Pharmacia. Uppsala. Sweden) as previously described (Hongo. J.S. et al., Hybridoma. *supra* (1995)). The purified antibody preparations were sterile
10   filtered (0.2-µm pore size; Nalgene. Rochester NY) and stored at 4 °C in phosphate buffered saline (PBS).

Expression of LIV-1 ECD Fragments in *E. coli*

Fragments of the LIV-1-164647 ECD were expressed in *E. coli* using the same techniques as described for cloning and expressing the full length LIV-1-164647. The expressed ECD fragments were the N-terminal fragment (amino acid 1 to and including amino acid 147 of SEQ ID NO:4) and the C-terminal fragment (amino
15   acid148 to and including amino acid 298 of SEQ ID NO:4). The fragments were purified as described above.

Standard Western blot analysis was used to determine which of the N-terminal or C-terminal ECD fragments bound to monoclonal antibodies isolated as described above. Only monoclonal antibodies 2945. 2982. 2984, 2985, 2987, and 2988 were tested. It was found that monoclonal antibody 2984 bound to the N-terminal fragment, while monoclonal antibody 2945. 2982, 2985, 2987, and 2988 bound to the C-terminal fragment. The
20   results are listed in Table 3.

Expression of Exogenous Full-Length LIV-1 in Mammalian Cells

Each of the 16 antibodies listed in Table 3 was examined for binding to the ECD of LIV-1-164647 and found to bind specifically. The antibodies were characterized to have the properties listed in Table 3. Epitope characterization involved determination as to whether a test antibody can compete for binding to the same epitope
25   as the epitope bound by an anti-LIV-1-164647 antibody of the present invention. including the antibodies produced by the hybridomas deposited with the ATCC, using a cross-blocking (e.g., a competitive ELISA assay) can be performed. In an exemplary competitive ELISA assay, LIV-1-167647 or its ECD (or other fragment) coated on the wells of a microtiter plate is pre-incubated with or without candidate competing antibody and then the biotin-labeled anti-LIV-1-164647 antibody of the invention is added. The amount of labeled anti-LIV-1-164647 antibody
30   bound to the LIV-1 antigen in the wells is measured using avidin-peroxidase conjugate and appropriate substrate. The antibody can be labeled with a radioactive or fluorescent label or some other detectable and measurable label. The amount of labeled anti-LIV-1 antibody bound to the antigen has an indirect correlation to the ability of the candidate competing antibody (test antibody) to compete for binding to the same epitope (*i.e.*, the greater the affinity of the test antibody for the same epitope, the less of the labeled antibody will be bound to the antigen-coated
35   wells). A candidate competing antibody is considered an antibody that binds substantially to the same epitope or that competes for binding to the same epitope as an anti-LIV-1 antibody of the invention if the candidate antibody can block binding of the LIV-1 antibody by at least 20%, preferably by at least 20-50%, even more preferably. by

at least 50% as compared to the control performed in parallel in the absence of the candidate competing antibody (but may be in the presence of a known non-competing antibody). It will be understood that variations of this assay can be performed to arrive at the same quantitative value. The epitope group assignment for each antibody in Table 3 was determined by competitive ELISA. Each antibody was biotinylated and tested for binding to LIV-1-164647

5   ECD coated on plates in the presence or absence of an excess of each unlabeled anti-LIV-1-164647 monoclonal antibody. Streptavidin-HRP was then added to the plates followed by peroxidase substrate. A decrease in the binding by at least 50% or a lack of binding of biotinylated monoclonal antibodies to LIV-1-164647 ECD indicated that both unlabeled and biotinylated antibodies bound to the same (or proximal) epitope on LIV-1-164647.

Expression of full-length LIV-1 in mammalian cells resulted in the exposure of the extracellular domain

10   on the surface of the cells. To demonstrate this, 3T3 cells were transiently transfected with an expression construct encoding wild-type full length LIV-1646471 (pRK5-LIV-1-164647). Control cells were transfected with the pRK5 vector lacking the LIV-1 insertion. After 24 hours, cells were analyzed for cell surface expression by fluorescent activated cell sorting (FACS) analysis using the anti-LIV-1-164647 ECD monoclonal antibody, described herein. as a tag. Approximately 10^6 cells were incubated for 30 min on ice in PBS containing 2% Goat serum and 5%

15   Rabbit serum (FACS buffer) then for 2 hours in FACS buffer containing 1μg/ml of anti-LIV-1-164647 ECD monoclonal antibody 2982 (isolated from hybridoma 2982.4A12.1E8.1C4, designated ATCC_____) or anti-LIV-1-164647 ECD monoclonal antibody 2983 (isolated from hybridoma 2983.3G9.1D4.1D7. ATCC_____) . The cells were then washed with ice cold PBS, incubated for 20 minutes at 4 °C with a Biotin-conjugated Goat anti-human IgG second antibody (Jackson Immunoreagents; West Grove. PA). washed before incubation for 20 min at

20   4 °C with phycoerythrin conjugated streptavidin (Jackson Immunoreagents; West Grove, PA). Cells were washed again prior to cytofluorometry. The FACS analysis results using antibody 2983 are plotted in Fig. 6. The results demonstrated that cell surface expression of LIV-1-164647 was detected in 3T3 cells transfected with pRK5- LIV-1-164647, but not in control (pRK5 vector only) cells. Thus, amino acids 1-298 of the LIV-1-164647 protein constitute an extracellular domain.

25   **Expression of Endogenous LIV-1 in a Breast Tumor Cell Line**

It is disclosed herein that endogenous LIV-1 is expressed on the surface of cells of the MCF-7 breast tumor cell line (ATCC HTB-22. for example). Anti-LIV-1 monoclonal antibody 2945 (from hybridoma 2945.2G1.1C7.2F10: ATCC_____) was radiolabeled with ^{125}I using the lactoperoxidase method. MCF-7 cells were harvested with PBS containing 1mM EDTA. A population of 3.4 million MCF-7 cells were incubated in PBSA

30   (PBS + 0.1% BSA + 0.02% azide) with 50 000 cpm (230pM) of ^{125}I-2945 in the presence (NSB) or absence (Tot) of an excess of unlabeled antibody (0.25μM) for 1h at room temperature. Bound radioactivity was then separated from unbound radioactivity by centrifugation for 5 min at 5000 rpm over a 1ml cushion of 20% sucrose in cold PBS. Radioactivity associated with the cell pellet was then counted in a gamma counter. As shown in Fig. 7. specific binding of anti-LIV-1-164647 monoclonal antibody 2945 was detected at the surface of MCF-7 cells indicating that

35   endogenous LIV-1 is expressed at the cell surface in this breast tumor cell line.

Antibody Binding to LIV-1

Selected monoclonal antibodies of the present invention and their respective hybridomas are listed in Table 3. Characterization of the monoclonal antibodies was performed as described herein using standard techniques of competition binding for epitope analysis. cell sorting for FACS analysis, and Western blotting for determining whether the antibody bound to the N-terminal or C-terminal portion of the LIV-1-164647 ECD.

Table 3

LIV-1-Binding Monoclonal Antibodies

Monoclonal Antibody	Hybridoma Cell Line	Isotype	Epitope Group[a]	FACS (3T3-LIV-1/3T3)	ECD fragment N- or C-terminal[b]
2945	2G1.1C7.2F10	IgG2b	A	+++/-	C-terminal
2982	4A12.1E8.1C4	IgG1	A	+++/-	C-terminal
2983	3G9.1D4.1D7	IgG1	A	+++/-	not tested
2984	6D6.1H10.2C1	IgG1	C	+++/-	N-terminal
2985	4F3.2D6.1D7	IgG1	A	+/-	C-terminal
2987	1D8.1C11.2B7	IgG1	C	++/-	C-terminal
2988	1A7.1F2.1H7	IgG1	A	+/-	C-terminal
2946	2B11.2B11.2A12	IgG2b	B	-/-	not tested
2947	3H8.2D9.1H8	IgG1	B	++/-	"
2948	2G4.2C7.2D6	IgG2b	B	-/-	"
2949	5B4.2H11.1G10	IgG2b	B	-/-	"
2950	5H5.2A7.1D8	IgG2b	B	-/-	"
2951	4G3.2F8.2A11	IgG2b	B	++/-	"
2952	6G9.1G9.1A10	IgG2b	B	-/-	"
2953	6B6.2E11.1F10	IgG1	B	+/-	"
2986	1F1.2G8.2E7	IgG1	B	-/-	"

[a] Epitope group assignments were made by antibody competition analysis as described herein.

[b] The term "N- or C-terminal" refers to binding of the tested antibody to the N-terminal or C-terminal fragment of the LIV-1-164647 ECD, where the N-terminal fragment was amino acid 1-147 of SEQ ID NO:4, and the C-terminal fragment was amino acids 148-298 of SEQ ID NO:4.

Deposit of some of these hybridomas with the American Type Culture Collection under the Budapest Treaty is described herein under the heading "Deposit of Material."

Example 5.   LIV-1 Expression in Tumor Tissue Examined by RNA *in situ* Hybridization

This example provides methods used in the preparation of tissue arrays for the determination of LIV-1 expression in various human tissues (see, for example, Kononen, J., et al. Nature Medicine 4:844-847 (1998)).

Preparation of Tissue Microarrays

A tissue microarray. or tissue array, is a paraffin block containing several individual tissue samples. A typical tissue microarray may contain 1000 or more samples. Tissue microarrays allow the examination of a large series of specimens while maximizing efficient utilization of technician time, reagents, and valuable tissue resources.

Tissue microarrays are constructed by first removing small cores (0.6mm diameter, 3-4 mm height) from "donor" tissue biopsy samples embedded in paraffin blocks using a tissue array instrument (Beecher Instruments, Silver Spring, MD, USA). Using the same instrument, each core sample is then re-embedded, together with other biopsy cores, in a single "recipient" block to form an array. Preferably, each tissue is sampled in triplicate. Thin

5　slices (4 - 8 µm thick) of a recipient block were mounted on glass slides. Visualization and screening may be performed by histological methods including, but not limited to, standard hematoxylin and eosin staining for morphological analysis; immunohistochemitry (IHC) for protein detection; mRNA *in situ* hybridization (mRNA ISH) and RT-PCR for mRNA detection; fluorescence in situ hybridization (FISH) and *in situ* PCR for DNA detection; and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay for

10　detection of cells undergoing apoptotic DNA fragmentation.

The following mRNA ISH procedures were used to determine a human tissue expression profile for LIV-1.

In situ Hybridization

In situ hybridization is a powerful and versatile technique for the detection and localization of nucleic acid

15　sequences within cell or tissue preparations. It may be useful, for example, to identify sites of gene expression, analyze the tissue distribution of transcription, identify and localize viral infection, follow changes in specific mRNA synthesis and aid in chromosome mapping.

In situ hybridization was performed following an optimized version of the protocol by Lu and Gillett, Cell Vision 1:169-176 (1994), using PCR-generated ^{33}P-labeled riboprobes. Briefly, formalin-fixed, paraffin-embedded

20　human tissues were sectioned, deparaffinized, deproteinated in proteinase K (20 µg/ml) for 15 minutes at 37°C, and further processed for *in situ* hybridization as described by Lu and Gillett, supra. A [33]-P] UTP-labeled antisense riboprobe was generated from a PCR product and hybridized at 55°C overnight. The slides were dipped in Kodak NTB2 nuclear track emulsion and exposed for 4 weeks.

Preparation of DNA template

25　For synthesis of a LIV-1-specific riboprobe, template DNA corresponding to a portion of LIV-1 was needed. To that end, a portion of the DNA 164647 (LIV-1-164647) nucleic acid sequence from nucleotide 1690 - nucleotide 2240 (SEQ ID NO:3; see Fig. 2A) having the following sequence was amplified for use as template DNA: 5'-TGCCATTCAC ATTTCCACGA TACACTCGGC CAGTCAGACG ATCTCATTCA CCACCATCAT GACTACCATC ATATTCTCCA TCATCACCAC CACCAAAACC ACCATCCTCA CAGTCACAGC

30　CAGCGCTACT CTCGGGAGGA GCTGAAAGAT GCCGGCGTCG CCACTTTGGC CTGGATGGTG ATAATGGGTG ATGGCCTGCA CAATTTCAGC GATGGCCTAG CAATTGGTGC TGCTTTTACT GAAGGCTTAT CAAGTGGTTT AAGTACTTCT GTTGCTGTGT TCTGTCATGA GTTGCCTCAT GAATTAGGTG ACTTTGCTGT TCTACTAAAG GCTGACATGA CCGTTAAGCA GGCTGTCCTT TATAATGCAT TGTCAGCCAT GCTGGCGTAT CTTGGAATGG CAACAGGAAT TTTCATTGGT

35　CATTATGCTG AAAATGTTTC TATGTGGATA TTTGCACTTA CTGCTGGCTT ATTCATGTAT GTTGCTCTGG TTGATATGGT ACCTGAAATG CTGCACAATG ATGCTAGTGA CCATGGATGT

AGCCGCTGGG G-3'(SEQ ID NO: 12). The amplified DNA was used as the template from which a radiolabeled riboprobe was synthesized for *in situ* hybridization.

Primers used for generating an antisense cDNA strand of LIV-1 from template nt 1690-nt 2240:

Primer F-99104: 5'- GGA TTC TAA TAC GAC TCA CTA TAG GGC TGC CAT TCA CAT TTC CAC

5   GAT-3'(SEQ ID NO:13).

Primer F-99105: 5'-CTA TGA AAT TAA CCC TCA CTA AAG GGA CCC CAG CGC CTA CAT CC-3'
(SEQ ID NO:14).

The Advantage cDNA polymerase mix from Clonetech (8417-1) was used according to the manufacturer's directions with slight modifications. Briefly. 316 µl SQ water (highly purified. RNase-free water), 40 µl 10 X PCR

10   buffer, 16 µl 10 mM dNT). 8 µl primer SEQ ID NO: 13, 8 µl primer SEQ ID NO: 14 were combined to form a master mixture. From the master mixture, 97 µl were aliquoted into a PCR tube followed by the addition of 2 µl of template DNA and 1 µl of Advantage cDNA polymerase. Using a Perkin-Elmer Cetus 9600 thermocycler. cycle conditions were as follows:

Begin: 85 ° C. 5 minutes

15   60 ° C. 1.5 minutes

10 cycles of:

94° C 30 seconds

68° C 30 seconds

20   72° C 1 minutes

15 cycles of:

94° C 30 seconds

55° C 30 seconds

25   72° C 1 minutes

followed by:

72° C 7 minutes

4° C hold

30

Upon completion of the PCR cycles, the PCR product was filter through a Microcon-50™ filter unit to remove primers and excess buffer.

LIV-1 ^{33}P-Riboprobe Synthesis

6.0 µl (125 mCi) of ^{33}P-UTP (Amersham BF 1002. SA<2000 Ci/mmol) were speed vac dried. To each

35   tube containing dried ^{33}P-UTP, the following ingredients were added:

2.0 µl 5x transcription buffer

1.0 µl DTT (100 mM)

2.0 µl NTP mix (2.5 mM: 10 µl; each of 10 mM GTP, CTP & ATP + 10 µl H$_2$O)

1.0 µl Rnasin ribonuclease inhibitor

3.0 µl DNA template (1 µg) in H$_2$O

1.0 µl RNA polymerase (for PCR products T3 = AS (antisense), T7 = S (sense), usually)

The tubes were incubated at 37°C for one hour. 1.0 µl RQ1 DNase was added followed by incubation at 37°C for 15 minutes. 90 µl TE (10 mM Tris pH 7.6/1mM EDTA pH 8.0) were added, and a 1.0 µl aliquot of the mixture was pipetted onto DE81 paper. The remaining solution was loaded in a Microcon-50 ultrafiltration unit (Amicon, 42416); and spun 6 minutes using program 10 of a Heraeus Centrifuge 28RS. The filtration unit was inverted over a second tube and spun for 3 minutes using program 2. After the final recovery spin, 100 µl TE were added. 1 µl of the final product was pipetted on DE81 paper. The samples dotted onto DE81 paper before and after filtration were counted in 6 ml of Biofluor II in a Beckman LS 5000TD scintillation counter.

To verify its size, the probe was run on a TBE/urea gel. 1-3 µl of the filtered probe or 5 µl of RNA Molecular Weight Marker III (Boehringer Mannheim) were added to 3 µl of loading buffer. After heating on a 37°C heat block for three minutes, the probes were immediately placed on ice. The wells of gel were flushed, the samples loaded, and run at 180-250 volts for 45 minutes. The gel was wrapped in saran wrap and exposed to Biomax MS™ film or XAR-2™ film (Kodak) with an intensifying screen in -70°C freezer one hour to overnight. The LIV-1 riboprobe thus prepared was designated 764 AS or 764 S for the antisense and sense probes, respectively.

[33]P-Hybridization

Pretreatment of paraffin-embedded sections: The thin slices of a recipient tissue array block mounted on glass slides were deparaffinized, placed in SQ H$_2$O, and rinsed twice in 2 x SSC at room temperature, for 5 minutes each time. The sections were deproteinated in 20 µg/ml proteinase K (500 µl of 10 mg/ml in 250 ml RNase-free RNase buffer) at 37°C for 15 minutes). Slides were subsequently rinsed in 0.5 x SSC, dehydrated through graded ethanols (70%, 95%, and 100%) for 2 minutes at each grade, and air-dryed.

Prehybridization: The slides were laid out in plastic box lined with Box buffer (4 x SSC, 50% formamide) - saturated filter paper. The tissue was covered with 100 µl of hybridization buffer (10% Dextran Sulfate, 50% formamide, 1XSSC) and incubated at 42°C for 1-4 hours.

Hybridization: 2.0 x 10⁶ cpm probe and 2.0 µl tRNA (100 mg/ml stock) per slide were heated at 95°C for 3 minutes. The slides were cooled on ice, and hybridization buffer was added to make a final volume of 100 µl per slide. After vortexing, 100 µl ^{33}P mix were added to 100 µl prehybridization on slide. The slides were incubated overnight at 55°C.

Washes: Washing was done 2x10 minutes with 2xSSC, EDTA at room temperature (400 ml 20 x SSC + 16 ml 0.25M EDTA, V$_f$=4L), followed by Rnase treatment at 37°C for 30 minutes (500 µl of 10 mg/ml in 250 ml prewarmed Rnase buffer = 20 µg/ml). The slides were washed 2x10 minutes with 2xSSC, EDTA at room temperature. The stringency wash conditions were as follows: 4 x 30 minutes at 55°C, 0.1 x SSC, EDTA (20 ml 20 x SSC + 16 ml EDTA, V$_f$=4L). This was followed by 2 x 10 minute washes 0.5SSc at RT. The slides were then dehydrated for 2 minutes at each of 50%, 70%, 90% ethanol contain 0.3 M ammonium acetate, and air dried for 2

hours. The dried slides were exposed to Biomax MS film (Kodak) for 16 hours or Hyperfilm β-Max film (Amersham) over 2 days.

ErbB2 and β-actin ^{33}P-Riboprobe Synthesis

For comparative analysis of LIV-1 and ErbB2 expression by RNA in situ hybridization. a riboprobe complementary to an ErbB2 nucleic acid sequence was prepared. As a control. expression of β-actin was also monitored using a riboprobe complementary to the RNA of that gene.

Using the procedures just disclosed. riboprobes specific for ErbB2 and β-actin were also prepared. The ErbB2 riboprobe was synthesized by transcription from a DNA template having the following sequence. 5'-TGGTCGTGGT CTTGGGGGTG GTCTTTGGGA TCCTCATCAA GCGACGGCAG CAGAAGATCC GGAAGTACAC GATGCGGAGA CTGCTGCAGG AAACGGAGCT GGTGGAGCCG CTGACACCTA GCGGAGCGAT GCCCAACCAG GCGCAGATGC GGATCCTGAA AGAGACGGAG CTGAGGAAGG TGAAGGTGCT TGGATCTGGC GCTTTTGGCA CAGTCTACAA GGGCATCTGG ATCCCTGATG GGGAGAATGT GAAAATTCCA GTGGCCATCA AAGTGTTGAG GGAAAACACA TCCCCCAAAG CCAACAAAGA AATCTTAGAC GAAGCATACG TGATGGCTGG TGTGGGCTCC CCATATGTCT CCCGCCTTCT GGGCATCTGC CTGACATCCA CGGTGCAGCT GGTGACACAG CTTATGCCCT ATGGCTGCCT CTTAGACCAT GTCCGGGAAA ACCGCGGACG CCTGGGCTCC CAGGACCTGC TGAACTGGTG TATGCAGATT GCCAAGGGGA TGAGCTACCT GGAGGATGTG CGGCTCGTAC ACAGGGACTT GGCCGCTCGG AACGTGCTGG TCAAGAGTCC CAACCATGTC AAAATTACAG ACTTCGGGCT GGCTCGGCTG-3' (SEQ ID NO:15). and its complement. The template also included a T7 and T3 promoter. The resultant ErbB2-specific antisense riboprobe was designated "442 AS."

The β-actin riboprobe was synthesized by transcription from a DNA template having the following sequence, 5'-GCTGCCTGAC GGCCAGGTCA TCACCATTGG CAATGAGCGG TTCCGCTGCC CTGAGGCACTCTTCCAGCCT TCCTTCCTGG GCATGGAGTC CTGTGGCATC CACGAAACTA CCTTCAACTC CATCATGAAG TGTGACGTG ACATCCGCAA AGACCTGTAC GCCAACACAG TGCTGTCTGG CGGCACCACC ATGTACCCTG GCATTGCCGA CAGGATGCAG AAGGAGATCA CTGCCCTGGC ACCCAGCACA ATGAAGATCA AGATCATTGC TCCTCTGAGC GCAAGTACTC-3' (SEQ ID NO:16), and its complement. The template included a T3 promoter. The resultant β-actin-specific antisense riboprobe was designated "117 AS."

Detection of LIV-1 in Tissue by RNA in situ Hybridization

This example provides results from tissue array analyses in which the expression of LIV-1 in various tissues was examined. Briefly, LIV-1 expression was found to occur in fetal kidney epithelium. developing fetal spinal ganglia including enteric plexuses, fetal brain, adult prostatic epithelium. breast tumors. breast fibroadenomas. lung carcinoma, squamous lung, colon carcinoma, prostate carcinoma. endometrial carcinoma, ovarian carcinoma. and melanoma.

Table 4 provides the results of several tissue microarray RNA in situ hybridization analyses. Unless otherwise indicated, the tissue microarrays were prepared as described herein. The tabulated results and comments were generated in studies designated IS2000-060 and IS2000-084 in which tissue microarrays were given section

numbers merely for internal reference. The relative expression of LIV-1 is indicated as "+", "++", or "+++" for increasing levels of detection, whereas no detectable expression was indicated as "-." The LIV-1 riboprobes used for RNA *in situ* hybridization were designated "764 S" as the sense probe and "764 AS" as the antisense probe. Antisense riboprobes specific for ErbB2 (probe 442 AS) and β-actin (probe 117 AS. as control) transcripts were also used in the IS2000-084 study. As used herein, the term "TMA" refers to tissue microarray. The term "NMA" refers to normal tissue microarray. where "normal" refers to non-cancerous tissue.

Table 4

Tissue Expression of LIV-1 by RNA *in situ* Hybridization

Tissue	Section[c]	Probe[a]	Result	Comments
Study ISH2000-060				
Control Cell Pellet	H2000-219.01	764 AS	-	
Control Cell Pellet	"	764 S	-	
LIV-1 Cell Pellet	H2000-219.02	764 AS	+++	
LIV-1 Cell Pellet	"	764 S	+	
Various, rhesus & human	Misc. 02	764 AS	-	
Various	Misc. 02	764 S	-	
12 wk Placenta	H97-039.02	764 AS	-	
12 wk Placenta	"	764 S	-	
14.5 wk Fetus	H97-106.31	764 AS	++	Expression in fetal kidney epithelium. developing spinal ganglia. including enteric plexuses
14.5 wk Fetus	"	764 S	-	
Fetal Brain	H97-045.01	764 AS	+	Expression over fetal cortical neurons
Fetal Brain	"	764 S	-	
Fetal Brain	H97-106.36	764 AS	+	
Adult NMA	H2000-165.06	764 AS	++	Strong expressioin over prostatic epithelium, focal low level expression over renal tubules, lung, gall bladder. spleen. heart and pancreas negative.
Adult NMA	"	764 S	-	
Breast TumorTMA	H2000-94B	764 AS	++	Expression elevated in 5 of 13 breast cancers; highly expressed in fibroadenomas
Tumor Block 3	H2000-165.20	764 AS	-	

Lung Tumor TMA	H1999-637	764 AS	+	Expression in one carcinoma and low level expression observed in normal bronchial epithelium
Lung Tumor TMA[b]	H2000-27	764 AS	++	Poor quality array. 6 cancers show moderate to high expression
Colon Tumor TMA	H1999-636	764 AS	+	Relative to breast. low level expression in 4 carcinomas
Colon Tumor TMA[b]	H2000-26	764 AS	-	Poor quality array
Breast Tumor TMA	H1999-635	764 AS	++	Expression observed in 3 carcinomas: highest levels seen in a case of fibroadenoma and one of sclerosing adenosis
Prostate Tumor TMA[b]	H2000-25	764 AS	++	At least 18 of the cases show reasonable signal: there is a reasonably strong signal in normal prostate
Human NMA	H2000-2	764 AS	+	Low level signal over adrenal cortex and renal tubular epithelium
Breast NMA	HP001595	764 AS	++	Expressed on nipple skin epithelium[d], breast ductal epithelium and acinar epithelium
Chimp NMA	H2000-185	764 AS	+	Expression over squamous epithelium
Multi-Tumor TMA	H2000-132	764 AS	+	Elevated expression in squamous lung. transitional. endometrial, ovarian carcinomas and melanoma

Study IS2000-084

Breast Tumor TMA	H2000-94	764 AS		Positive for LIV-1 mRNA
	"	442 AS		Positive for ErbB2 mRNA
	"	117 AS		Positive for β-actin mRNA

[a]Riboprobe preparation and number designations are disclosed in this Example.

[b]Array purchased from Clinomics Biosciences, Inc., Pittsfield, MA.

[c]Several of the sections listed in Table 4 contained various tissue types. For example, section Misc. 02 contained normal (i.e. non-cancerous) kidney. bladder. lung, and end stage renal disease (non-cancerous). Tumor block 3 (H2000-165.20) contained chondrosarcoma. osteosarcoma. renal cell carcinoma. liposarcoma. gastric adenocarcinoma, squamous carcinoma. and brain tumor. Human NMA (H2000-2) contained samples of pancreas, adrenal. heart, eye, small intestine. kidney. spleen. lymph node. tonsil, skin. breast. lung. brain. colon. liver, aorta.

placenta. stomach. ovary. prostate. breast, and skin. All lymph node specimens on H2000-2 were actin negative suggesting that mRNA was degraded in these samples. whereas heart samples were only weakly actin positive). Breast TMA (section H2000-94B) contained samples of atypical periductal stromal proliferation (previous excision of cystosarcoma). reactive changes with giant cells; DCIS (0 of 4 lymph node (LN) positive): Ductal carcinoma.

5   invasive; Ductal carcinoma. poorly-differentiated, invasive (0 of 24 LN positive): Atypical hyperplasia/CIS : Carcinoma with ductal and lobular features, infiltrative. lymph node metastasis; Ductal carcinoma, invasive. grade III/III; DCIS. low grade; LCIS (0 of 26 LN pos); Benign breast tissue; Fibroadenoma; Adenocarcinoma, invasive (31 of 31 LN pos); Adenosis. Multi tumor TMA (section H2000-132) contained samples from lung tumor (bronchioloalveolar carcinoma, adenocarcinoma); colon adenocarcinoma; gastric adenocarcinoma; pancreatic ductal

10   adenocarcinoma; heptocellular carcinoma; prostate adenocarcinoma (Gleason grade 2-3); bladder transitional cell carcinoma; kindney papillary transitional cell carcinoma; prostate transitional cell carcinoma; renal clear cell carcinoma; endometrial adenocarcinoma; ovary papillary adenocarcinoma; ovary clear cell adenocarcinoma; lymphoma; and melanoma.

         [d] Quantitative microarray analysis of extracted mRNA from cells of a different sample of normal breast

15   skin indicated low LIV-1 expression in skin relative to breast tumor.

         As the results of Study IS2000-060 in Table 4 indicate. RNA *in situ* hybridization confirms elevated expression of LIV-1 in some breast cancers relative to normal breast: expression was elevated in 5 of 13 breast cancers in a TMA. High expression was also seen in benign breast disease, specifically fibroadenomas and

20   sclerosing adenosis. Strong expression was observed in normal prostatic epithelium as well as in prostate cancers. Expression was seen in epithelium of a number of other tumor types including: squamous lung, transitional. endometrial. ovarian carcinomas and melanoma. With regard to expression in normal tissue. LIV-1 is moderately to highly expressed in normal squamous epithelium (*e.g.*. chimp and human breast skin). Strong expression was observed over normal prostatic epithelium, focal low level expression over normal renal tubules. Liver, lung. gall

25   bladder, spleen. heart, and pancreas were all negative for LIV-1 RNA expression. In fetus. expression was seen in fetal kidney epithelium as well as in developing spinal ganglia. including enteric plexuses and fetal brain.

         In Study IS2000-084, the relative expression of RNA of β-actin (as control). ErbB2. and LIV-1 in breast tumor were compared. The results are shown in Table 4. All samples exhibited adequate expression of β-actin RNA. Weak to moderate expression of ErbB2 (HER2) RNA was seen in benign and malignant epithelial cells of most

30   cases. Particularly strong expression was seen in four cases, three cases of infiltrating ductal carcinoma, and one case of ductal carcinoma *in situ* (DCIS). LIV-1 RNA was observed in the benign and malignant mammary epithelial cells in most cases at an intensity ranging from weak to strong. with most cases showing a moderate level of expression. There was no indication in the tissues sampled for this study that LIV-1 was up-regulated in the malignant cells compared to benign cells.

Example 6.   Detecting LIV-1 Expression in Cells

Diagnosis of breast tumor tissue as the type that overexpresses LIV-1 but does not simultaneously express ErbB2 is useful to allow the physician to tailor the patients tumor therapy.

Detection of LIV-1-164647 expression and ErbB2 expression in breast tumor (or any tumor or other cells)
5   is readily performed by microarray technology. as described in Example 1. coupled with ordinary knowledge of relevant microarray technology procedures.

Detection of LIV-1-164647 expression in a cell may be performed by in situ hybridization. where the probe for detecting is derived from the ECD of LIV-1-164647 and is a cDNA or a RNA having a sequence that hybridizes under stringent conditions to a sequence from nucleotide 412 to and including nucleotide 477 of SEQ ID NO:3 or
10   its complementary sequence. Preferably, the probe hybridizes to a sequence from nucleotide 446 to and including nucleotide 464 of SEQ ID NO:3 or its complementary sequence. Hybridization protocols useful for this method of detection are standard in the relevant literature. A non-limiting RNA *in situ* hybridization technique useful for detecting LIV-1-164647 expression is disclosed herein.

Alternatively, relative expression of LIV-1 and ErbB2 is performed by contacting an anti-LIV-1-164647
15   antibody of the invention that specifically binds to the extracellular domain of LIV-1-164647 protein and comparing the amount of detectable binding with control cells that do not express LIV-1-164647 protein; with cells (such as SKBR3 cells) that overexpress ErbB2, but do not overexpress LIV-1-164647; and with cells (such as cells expressing LIV-1 or DNA164647) that overexpress LIV-1-164647 but do not overexpress ErbB2, wherein overexpression is determined as at least 1.5-fold greater expression in a cell from tumor tissue relative to expression
20   in a cell from non-cancerous tissue. The techniques for binding anti-LIV-1 antibody and/or anti-ErbB2 antibody are readily determined based on disclosure provided herein coupled with ordinary skill in the art of cell surface protein detection. Preferably, the antibody used for detecting expression of the LIV-1-164647 polypeptide binds to the ECD of LIV-1-164647, preferably binding to epitope A, epitope B, or epitope C of an LIV-1 ECD, preferably the LIV-1-164647 ECD. Alternatively the antibody used for detecting expression of an LIV-1 polypeptide binds to a
25   polypeptide comprising an amino acid sequence from amino acid 114 to and including amino acid 135 of SEQ ID NO:4, more preferably comprising a sequence from amino acid 126 to and including 132 of SEQ ID NO:4. Antibodies useful for practicing this method are described herein and include without limitation the monoclonal antibodies produced by one or more of the hybridomas ATCC_____ (LIV-1.2945.2G1.1C7.2F10); ATCC _____ (LIV-1.2982.4A12.1E8.1C4); ATCC _____ (LIV-1.2983.3G9.1D4.1D7); ATCC _____ (LIV-
30   1.2984.6D6.1H10.2C1); ATCC _____ (LIV-1.2985.4F3.2D6.1D7); ATCC _____ (LIV-1.2987.1D8.1C11.2B7); and ATCC _____ (LIV-1.2988.1A7.1F2.1H7). Techniques useful for performing antibody binding studies are disclosed herein and are found in the relevant literature.

Example 7.   Pharmaceutical Formulations

Antibodies specifically binding a LIV-1 polypeptide of the invention. or a fragment of the LIV-1
35   polypeptide, such as the ECD, which may be identified by the screening assays disclosed herein. can be administered for the treatment of tumors, including cancers. in the form of pharmaceutical compositions.

Where antibody fragments are used, the smallest inhibitory fragment which specifically binds to the binding domain of the target protein is preferred. For example, based upon the variable region sequences of an antibody, peptide molecules can be designed which retain the ability to bind the target protein sequence. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology (see. *e.g.* Marasco *et al.*, Proc. Natl.

5   Acad. Sci. USA 90, 7889-7893 [1993]). If the antibody that binds a LIV-1 protein binds to an intracellular portion, and whole antibodies or fragments are used as inhibitors, internalizing the antibodies is preferred. Lipofections or liposomes can be used to deliver the antibody, or an antibody fragment, into cells.

Therapeutic formulations of the antibodies used in accordance with the present invention are prepared for storage by mixing an antibody having the desired degree of purity with optional pharmaceutically acceptable

10   carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition. Osol, A. Ed. [1980]), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or

15   benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone: amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins: chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose

20   or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes): and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide antibodies which bind to vascular endothelial factor (VEGF) in the

25   one formulation. Alternatively, or in addition, the composition may comprise a cytotoxic agent, cytokine or growth inhibitory agent, provided that the cytotoxic agent is other than an anthracycline derivative, *e.g.* doxorubicin, or epirubicin. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation

30   techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by

35   filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the

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form of shaped articles. e.g. films. or microcapsules. Examples of sustained-release matrices include polyesters. hydrogels (for example. poly(2-hydroxyethyl-methacrylate). or poly(vinylalcohol)). polylactides (U.S. Pat. No. 3,773.919), copolymers of L-glutamic acid and γ ethyl-L-glutamate. non-degradable ethylene-vinyl acetate. degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed

5   of lactic acid-glycolic acid copolymer and leuprolide acetate). and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days. certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C. resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization

10   depending on the mechanism involved. For example. if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange. stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions. controlling moisture content, using appropriate additives. and developing specific polymer matrix compositions.

Example 8.   Articles of Manufacture

15   In another embodiment of the invention, an article of manufacture containing materials useful for the diagnosis or treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes. and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for detecting (e.g., diagnosing) or treating the condition and may have a sterile access port (for

20   example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is usually an anti-tumor agent capable of interfering with the activity of a gene product identified herein, e.g. an antibody. The label on, or associated with, the container indicates that the composition is used for diagnosing or treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer. such as phosphate-

25   buffered saline. Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers. diluents. filters, needles, syringes. and package inserts with instructions for use.

Example 9.   Diagnosis and Prognosis of Tumors

While cell surface proteins, such as growth receptors overexpressed in certain tumors are excellent

30   targets for drug candidates or tumor (e.g. cancer) treatment. the same proteins along with secreted proteins encoded by the genes amplified in tumor cells find additional use in the diagnosis and prognosis of tumors. For example. antibodies directed against the proteins products of genes amplified in tumor cells can be used as tumor diagnostics or prognostics.

For example, antibodies. including antibody fragments. can be used to qualitatively or quantitatively detect

35   the expression of proteins encoded by the amplified genes ("marker gene products"). The antibody preferably is equipped with a detectable, e.g. fluorescent label. and binding can be monitored by light microscopy, flow cytometry. fluorimetry. or other techniques known in the art. These techniques are particularly suitable. if the

amplified gene encodes a cell surface protein. *e.g.* a growth factor. Such binding assays are performed essentially as described herein.

In situ detection of antibody binding to the marker gene products can be performed. for example. by immunofluorescence or immunoelectron microscopy. For this purpose. a histological specimen is removed from the patient. and a labeled antibody is applied to it. preferably by overlaying the antibody on a biological sample. This procedure also allows for determining the distribution of the marker gene product in the tissue examined. It will be apparent for those skilled in the art that a wide variety of histological methods are readily available for *in situ* detection.

Example 10.   Determination of LIV-1 in tissue or body fluid

Described herein are serological methods for determining the presence of LIV-1 gene product (e.g. LIV-1-164647 protein or fragments thereof) in the body fluid of a mammal. preferably a human patient potentially suffering from the growth of cells overexpressing LIV-1. The method preferably detects the presence and. optionally. quantifies, the amount of LIV-1 extracellular domain or fragments thereof in serum of such a patient. thereby providing a relatively non invasive method of detecting the overexpression of LIV-1 in a patient.

Essentially. the processes of this embodiment of the invention comprise incubating or otherwise exposing a sample of body fluid potentially containing LIV-1-164647 extracellular domain or fragments thereof. to anti-LIV-1-164647 monoclonal antibodies and detecting the presence of a reaction product. Those skilled in the art will recognize that there are many variations of these basic procedures. These include. for example, RIA, ELISA, precipitation. agglutination. complement fixation and immuno-fluorescence. In the currently preferred procedures, the monoclonal antibodies are appropriately labeled for detection. Labels useful in the practice of the invention include. but are not limited to. moieties, such as enzymes. that must be reacted or derivatized to be detected. The enzyme label can be detected by any of the currently utilized colorimetric, spectrophotometric, fluorospectrophotmetric or gasometric techniques. The enzyme is combined with the antibody with bridging molecules such as carbodiimides, periodate, diisocyanates. glutaraldehyde and the like. Many enzymes which can be used in these procedures are known and can be utilized. Examples are peroxidase. alkaline phosphatase. β-glucuronidase. β-D-glucosidase. β-D-galactosidase. urease. glucose oxidase plus peroxidase. galactose oxidase plus peroxidase and acid phosphatase. Fluorescent materials which may be used include. for example. fluorescein and its derivatives. rhodamine and its derivatives, auramine. dansyl. umbelliferone, luciferia, 2,3-dihydrophthalazinediones. horseradish peroxidase, alkaline phosphatase. lysozyme. and glucose-6-phosphate dehydrogenase. The antibodies may be tagged with such labels by known methods. For instance, coupling agents such as aldehydes, carbodiimides. dimaleimide, imidates, succinimides. bid-diazotized benzadine and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. Various labeling techniques are described in Morrison, Methods in Enzymology 32b:103 [1974]; Syvanen et al., J. Biol. Chem. 284:3762 [1973]; and Bolton and Hunter. Biochem J. 133:529 [1973]. Additionally, a radiolabelled antibody can be detected by any of the currently available counting procedures. Preferred isotope labels are ^{99}Tc, ^{14}C, ^{131}I, ^{125}I, ^3H, ^{32}P, and ^{35}S.

Additionally, the following non-limiting assay is useful for determining the presence of and to quantitate the amount of specific anti-LIV-1 monoclonal antibody (preferably specific to the extracellular domain of the LIV-1-164647 gene product, or a portion of the extracellular domain) in a body fluid of a mammal. The body fluid may include, but is not limited to serum, amniotic fluid, milk, umbilical cord serum, ocular aqueous and vitreous liquids,

5   and ocular vitreous gel.

Plate Binding Activity Assay Using Humanized Anti-LIV-1 Monoclonal Antibody. The method of assaying anti-LIV-1 antibody described herein is meant as an example of such a method and is not meant to be limiting. A standardized preparation of anti-LIV-1 antibody, preferably specific to the extracellular domain of the LIV-1-167647 gene product, controls and serum samples are diluted with Assay Diluent (PBS/0.5% BSA/0.05%

10   Polysorbate 20/0.01% Thimerosal). The dilutions of standardized anti-LIV-1 antibody are prepared to span a range of concentrations useful for a standard curve. The samples are diluted to fall within the standard curve.

An aliquot of Coat Antigen in Coating buffer (anti-LIV-1-164647 antibody in 0.05 M sodium carbonate buffer) is added to each well of a microtiter plate and incubated at 2-8°C for 12-72 hours. The coating solution is removed and each well is washed six times with water, then blotted to remove excess water. An aliquot of Assay

15   Diluent is added to each well and incubated for 1-2 hours at ambient temperature with agitation. The wells are washed as in the previous step. Aliquots of diluted standard, control and sample solutions are added to the wells and incubated at ambient temperature for 1 hour with agitation to allow binding of the antibody to the coating antigen. The wells are washed again with water as in previous steps.

Horse radish peroxidase-conjugate (HRP-conjugate, Goat anti-human IgG Fc conjugated to horseradish

20   peroxidase, (Organon Teknika catalog #55253 or equivalent) is diluted with Assay Diluent to yield an appropriate optical density range between the highest and lowest standards. An aliquot of the HRP-conjugate solution is added to each well and incubated at ambient temperature for 1 hour with agitation. The wells are washed with water as in previous steps.

An aliquot of Substrate Solution (o-phenylenediamine (OPD) 5 mg tablet (Sigma P6912 or equivalent) in

25   12.5 ml 4 mM H_2O_2 in PBS) is added to each well and incubated for a sufficient period of time (approximately 8-10 minutes) in the dark at ambient temperature to allow color development. The reaction is stopped with an aliquot of 4.5 N sulfuric acid. Optical density is read at 490-492 nm for detection absorbance and 405 nm for reference absorbance. The standard curve data are plotted and the results for the controls and samples are determined from the standard curve.

30   **Example 11.   Methods of Treatment**

It is contemplated that, according to the present invention, the anti-LIV-1 antibodies or other LIV-1 activity-blocking molecules may be used to treat various conditions characterized by overexpression and/or activation of the LIV-1 gene product with or without coexpression of ErbB2 above the ErbB2 expression found in healthy, nonmalignant cells. Exemplary conditions or disorders to be treated with such antibodies and other

35   compounds, including, but not limited to, small organic and inorganic molecules, peptides, antisense molecules, etc. include benign or malignant tumors (e.g. breast, prostate, lung, and colon, as well as renal, liver, kidney, bladder, gastric, ovarian, colorectal, pancreatic, vulval, thyroid, hepatic carcinomas; sarcomas; glioblastomas; and various

head and neck tumors); leukemias and lymphoid malignancies; other disorders such as neuronal. glial. astrocytal. hypothalamic and other glandular. macrophagal, epithelial. stromal and blastocoelic disorders; and inflammatory. angiogenic and immunologic disorders. Where an antibody is used to treat an LIV-1 overexpression -related disorder, the antibody is preferably an anti-LIV-1-164647 antibody, more preferably a humanized antibody which

5   binds to a polypeptide comprising an amino acid sequence from amino acid 114 to and including amino acid 135 of SEQ ID NO:4.

The anti-tumor agents of the present invention, *e.g.* antibodies, are administered to a mammal, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal. subcutaneous. intra-articular,

10   intrasynovial, intrathecal, oral, topical. or inhalation routes. Intravenous administration of the antibody is preferred.

Other therapeutic regimens may be combined with the administration of the anti-cancer agents, *e.g.* antibodies of the instant invention. For example, the patient to be treated with such anti-cancer agents may also receive radiation therapy. Alternatively, or in addition, a chemotherapeutic agent may be administered to the patient. Preparation and dosing schedules for such chemotherapeutic agents may be used according to

15   manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in *Chemotherapy Service* Ed., M.C. Perry, Williams & Wilkins, Baltimore, MD (1992). The chemotherapeutic agent may precede, or follow administration of the anti-tumor agent, *e.g.* antibody, or may be given simultaneously therewith. The antibody may be combined with an anti-oestrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see, EP 616812) in dosages known for

20   such molecules.

It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to the ErbB2, EGFR. ErbB3, ErbB4, or vascular endothelial factor (VEGF). Alternatively, or in addition, two or more antibodies binding the same or two or more different antigens disclosed herein may be co-administered to the patient. Sometimes, it may be beneficial to also administer one or more cytokines to the patient.

25   In a preferred embodiment, the antibodies herein are co-administered with a growth inhibitory agent. For example, the growth inhibitory agent may be administered first, followed by an antibody of the present invention. However, simultaneous administration or administration of the antibody of the present invention first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the growth inhibitory agent and the antibody herein.

30   Where combined administration of a chemotherapeutic agent is desired. the combined administration includes co-administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities. Preparation and dosing schedules for such chemotherapeutic agents may be used according to manufacturers' instructions or as determined empirically by the skilled practitioner. Preparation and dosing schedules for such chemotherapy are also described in Chemotherapy Service Ed., M.C.

35   Perry, Williams & Wilkins, Baltimore, MD (1992). The chemotherapeutic agent may precede, or follow administration of the antibody or may be given simultaneously therewith. The antibody may be combined with an

anti-estrogen compound such as tamoxifen or an anti-progesterone such as onapristone (see. EP 616 812) in dosages known for such molecules.

    It may be desirable to also administer antibodies against other tumor associated antigens, such as antibodies which bind to vascular endothelial factor (VEGF). Alternatively. or in addition, two or more anti-LIV-1 antibodies

5   may be co-administered to the patient. Sometimes. it may be beneficial to also administer one or more cytokines to the patient. The anti-LIV-1 antibody may be co-administered with a growth inhibitory agent. For example. the growth inhibitory agent may be administered first, followed by the anti-LIV-1 antibody. However, simultaneous administration or administration of the anti-LIV-1 antibody first is also contemplated. Suitable dosages for the growth inhibitory agent are those presently used and may be lowered due to the combined action (synergy) of the

10   growth inhibitory agent and anti-LIV-1 antibody.

    In addition to the above therapeutic regimens. the patient may be subjected to surgical removal of cancer cells and/or radiation therapy.

    For example, depending on the type and severity of the disease. about 1 µg/kg to 15 mg/kg (e.g. 0.1-20mg/kg) of antibody is an initial candidate dosage for administration to the patient. whether. for example, by one

15   or more separate administrations. or by continuous infusion. A typical daily dosage might range from about 1 µg/kg to 100 mg/kg or more. depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However. other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

20   **Example 12.   Antibody Binding Studies**

    The results of the gene amplification study can be further verified by antibody binding studies, in which the ability of anti-LIV-1-164647 antibodies to detect the presence of or to inhibit the effect of the LIV-1 polypeptides on tumor (cancer) cells is tested. Exemplary antibodies include polyclonal. monoclonal, humanized. bispecific. and heteroconjugate antibodies. the preparation of which will be described hereinbelow.

25   Antibody binding studies may be carried out in any known assay method. such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc., 1987).

    Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of target protein (encoded by a gene amplified in a

30   tumor cell) in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies preferably are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

    Sandwich assays involve the use of two antibodies. each capable of binding to a different immunogenic

35   portion. or epitope. of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, *e.g.*, US Pat No. 4.376,110. The second antibody may itself be

labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

For immunohistochemistry, the tumor sample may be fresh or frozen or may be embedded in paraffin and fixed with a preservative such as formalin, for example.

Example 13.   Cell-Based Tumor Assays

Cell-based assays and animal models for tumors (*e.g.* cancers) can be used to verify the findings of the gene amplification assay, and further understand the relationship between the genes identified herein and the development and pathogenesis of neoplastic cell growth. The role of gene products identified herein in the development and pathology of tumor or cancer can be tested by using primary tumor cells or cells lines that have been identified to amplify the genes herein. Such cells include, for example, the breast and prostate cancer cells and cell lines listed above.

In a different approach, cells of a cell type known to be involved in a particular tumor are transfected with the cDNAs herein, and the ability of these cDNAs to induce excessive growth is analyzed. Suitable cells include, for example, stable tumor cells lines such as, the B104-1-1 cell line (stable NIH-3T3 cell line transfected with the *neu* protooncogene) and *ras*-transfected NIH-3T3 cells, which can be transfected with the desired gene, and monitored for tumorogenic growth. Such transfected cell lines can then be used to test the ability of poly- or monoclonal antibodies or antibody compositions to inhibit tumorogenic cell growth by exerting cytostatic or cytotoxic activity on the growth of the transformed cells, or by mediating antibody-dependent cellular cytotoxicity (ADCC). Cells transfected with the coding sequences of the genes identified herein can further be used to identify drug candidates for the treatment of cancer.

In addition, primary cultures derived from tumors in transgenic animals (as described below) can be used in the cell-based assays herein, although stable cell lines are preferred. Techniques to derive continuous cell lines from transgenic animals are well known in the art (see, *e.g.* Small *et al.*, Mol. Cell. Biol. 5, 642-648 [1985]).

Example 14.   Animal Models

A variety of well known animal models can be used to further understand the role of the genes identified herein in the development and pathogenesis of tumors, and to test the efficacy of candidate therapeutic agents, including antibodies, and other antagonists of the native polypeptides, including small molecule antagonists. The *in vivo* nature of such models makes them particularly predictive of responses in human patients. Animal models of tumors and cancers (*e.g.* breast cancer, colon cancer, prostate cancer, lung cancer, etc.) include both non-recombinant and recombinant (transgenic) animals. Non-recombinant animal models include, for example, rodent, *e.g.*, murine models. Such models can be generated by introducing tumor cells into syngeneic mice using standard techniques, *e.g.* subcutaneous injection, tail vein injection, spleen implantation, intraperitoneal implantation, implantation under the renal capsule, or orthopin implantation, *e.g.* colon cancer cells implanted in colonic tissue. (See, *e.g.* PCT publication No. WO 97/33551, published September 18, 1997).

Probably the most often used animal species in oncological studies are immunodeficient mice and, in particular, nude mice. The observation that the nude mouse with hypo/aplasia could successfully act as a host for

human tumor xenografts has lead to its widespread use for this purpose. The autosomal recessive *nu* gene has been introduced into a very large number of distinct congenic strains of nude mouse. including. for example. ASW. A/He. AKR, BALB/c, B10.LP, C17, C3H. C57BL. C57. CBA, DBA, DDD. I/st. NC. NFR. NFS. NFS/N, NZB. NZC. NZW, P, RIII and SJL. In addition. a wide variety of other animals with inherited immunological defects other than
5   the nude mouse have been bred and used as recipients of tumor xenografts. For further details see. *e.g. The Nude Mouse in Oncology Research*, E. Boven and B. Winograd, eds., CRC Press, Inc., 1991.

The cells introduced into such animals can be derived from known tumor/cancer cell lines, such as. any of the above-listed tumor cell lines, and. for example, the B104-1-1 cell line (stable NIH-3T3 cell line transfected with the *neu* protooncogene); *ras*-transfected NIH-3T3 cells; Caco-2 (ATCC HTB-37); a moderately well-
10   differentiated grade II human colon adenocarcinoma cell line. HT-29 (ATCC HTB-38). or from tumors and cancers. Samples of tumor or cancer cells can be obtained from patients undergoing surgery, using standard conditions, involving freezing and storing in liquid nitrogen (Karmali *et al.*, Br. J. Cancer 48, 689-696 [1983]). Tumor cells can be introduced into animals, such as nude mice. by a variety of procedures. The subcutaneous (s.c.) space in mice is very suitable for tumor implantation. Tumors can be transplanted s.c. as solid blocks. as needle biopsies by
15   use of a trochar, or as cell suspensions. For solid block or trochar implantation, tumor tissue fragments of suitable size are introduced into the s.c. space. Cell suspensions are freshly prepared from primary tumors or stable tumor cell lines, and injected subcutaneously. Tumor cells can also be injected as subdermal implants. In this location. the inoculum is deposited between the lower part of the dermal connective tissue and the s.c. tissue. Boven and Winograd (1991), *supra*.

20   Animal models of breast cancer can be generated, for example. by implanting rat neuroblastoma cells (from which the *neu* oncogen was initially isolated), or *neu* -transformed NIH-3T3 cells into nude mice, essentially as described by Drebin *et al.* PNAS USA 83, 9129-9133 (1986).

Similarly, animal models of colon cancer can be generated by passaging colon cancer cells in animals, *e.g.* nude mice, leading to the appearance of tumors in these animals. An orthotopic transplant model of human colon
25   cancer in nude mice has been described. for example, by Wang *et al.*, Cancer Research 54, 4726-4728 (1994) and Too *et al.*, Cancer Research 55, 681-684 (1995). This model is based on the so-called "METAMOUSE™" sold by AntiCancer, Inc. (San Diego, California).

Tumors that arise in animals can be removed and cultured *in vitro*. Cells from the *in vitro* cultures can then be passaged to animals. Such tumors can serve as targets for further testing or drug screening. Alternatively, the
30   tumors resulting from the passage can be isolated and RNA from pre-passage cells and cells isolated after one or more rounds of passage analyzed for differential expression of genes of interest. Such passaging techniques can be performed with any known tumor or cancer cell lines.

For example, Meth A, CMS4, CMS5. CMS21, and WEHI-164 are chemically induced fibrosarcomas of BALB/c female mice (DeLeo *et al.*, J. Exp. Med. 146, 720 [1977]), which provide a highly controllable model
35   system for studying the anti-tumor activities of various agents (Palladino *et al.*, J. Immunol. 138, 4023-4032 [1987]). Briefly, tumor cells are propagated *in vitro* in cell culture. Prior to injection into the animals, the cell lines

are washed and suspended in buffer. at a cell density of about 10×10^6 to 10×10^7 cells/ml. The animals are then infected subcutaneously with 10 to 100 µl of the cell suspension, allowing one to three weeks for a tumor to appear.

In addition. the Lewis lung (3LL) carcinoma of mice, which is one of the most thoroughly studied experimental tumors. can be used as an investigational tumor model. Efficacy in this tumor model has been
5   correlated with beneficial effects in the treatment of human patients diagnosed with small cell carcinoma of the lung (SCCL). This tumor can be introduced in normal mice upon injection of tumor fragments from an affected mouse or of cells maintained in culture (Zupi *et al.*. Br. J. Cancer 41, suppl. 4. 309 [1980]). and evidence indicates that tumors can be started from injection of even a single cell and that a very high proportion of infected tumor cells survive. For further information about this tumor model see Zacharski, Haemostasis 16. 300-320 [1986]).

10   One way of evaluating the efficacy of a test compound on an implanted tumor in an animal model is to measure the size of the tumor before and after treatment. Traditionally, the size of implanted tumors has been measured with a slide caliper in two or three dimensions. The measure limited to two dimensions does not accurately reflect the size of the tumor. therefore. it is usually converted into the corresponding volume by using a mathematical formula. However. the measurement of tumor size is very inaccurate. The therapeutic effects of
15   a drug candidate can be better described as treatment-induced growth delay and specific growth delay. Another important variable in the description of tumor growth is the tumor volume doubling time. Computer programs for the calculation and description of tumor growth are also available, such as the program reported by Rygaard and Spang-Thomsen, *Proc. 6th Int. Workshop on Immune-Deficient Animals*, Wu and Sheng eds., Basel. 1989. 301. It is noted, however, that necrosis and inflammatory responses following treatment may actually result in an increase
20   in tumor size, at least initially. Therefore. these changes need to be carefully monitored. by a combination of a morphometric method and flow cytometric analysis.

Recombinant (transgenic) animal models can be engineered by introducing the coding portion of the genes identified herein into the genome of animals of interest. using standard techniques for producing transgenic animals. Animals that can serve as a target for transgenic manipulation include. without limitation. mice, rats,
25   rabbits, guinea pigs. sheep. goats. pigs, and non-human primates. *e.g.* baboons. chimpanzees and monkeys. Techniques known in the art to introduce a transgene into such animals include pronucleic microinjection (Hoppe and Wanger, U.S. Patent No. 4.873,191); retrovirus-mediated gene transfer into germ lines (*e.g.*. Van der Putten *et al.*. Proc. Natl. Acad. Sci. USA 82. 6148-615 [1985]); gene targeting in embryonic stem cells (Thompson *et al.*. Cell 56. 313-321 [1989]); electroporation of embryos (Lo, Mol. Cel.. Biol. 3, 1803-1814 [1983]): sperm-mediated gene
30   transfer (Lavitrano *et al.*, Cell 57, 717-73 [1989]). For review, see, for example, U.S. Patent No. 4.736.866.

For the purpose of the present invention, transgenic animals include those that carry the transgene only in part of their cells ("mosaic animals"). The transgene can be integrated either as a single transgene. or in concatamers, *e.g.*. head-to-head or head-to-tail tandems. Selective introduction of a transgene into a particular cell type is also possible by following. for example, the technique of Lasko *et al.*. Proc. Natl. Acad. Sci. USA 89. 6232-
35   636 (1992).

The expression of the transgene in transgenic animals can be monitored by standard techniques. For example, Southern blot analysis or PCR amplification can be used to verify the integration of the transgene. The

92

level of mRNA expression can then be analyzed using techniques such as *in situ* hybridization, Northern blot analysis, PCR, or immunocytochemistry. The animals are further examined for signs of tumor or cancer development.

Alternatively, "knock out" animals can be constructed which have a defective or altered gene encoding a
5   LIV-1-164647 polypeptide identified herein, as a result of homologous recombination between the endogenous gene encoding the polypeptide and altered genomic DNA encoding the same polypeptide introduced into an embryonic cell of the animal. For example, cDNA encoding a particular LIV-1 polypeptide can be used to clone genomic DNA encoding that polypeptide in accordance with established techniques. A portion of the genomic DNA encoding a particular LIV-1 polypeptide can be deleted or replaced with another gene, such as a gene encoding a selectable
10   marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see *e.g.*, Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (*e.g.*, by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see *e.g.*, Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an
15   animal (*e.g.*, a mouse or rat) to form aggregation chimeras [see *e.g.*, Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously
20   recombined DNA. Knockout animals can be characterized for instance, by their ability to defend against certain pathological conditions and by their development of pathological conditions due to absence of the LIV-1 polypeptide.

The efficacy of antibodies specifically binding the polypeptides identified herein and other drug candidates, can be tested also in the treatment of spontaneous animal tumors. A suitable target for such studies is the feline oral
25   squamous cell carcinoma (SCC). Feline oral SCC is a highly invasive, malignant tumor that is the most common oral malignancy of cats, accounting for over 60% of the oral tumors reported in this species. It rarely metastasizes to distant sites, although this low incidence of metastasis may merely be a reflection of the short survival times for cats with this tumor. These tumors are usually not amenable to surgery, primarily because of the anatomy of the feline oral cavity. At present, there is no effective treatment for this tumor. Prior to entry into the study, each cat
30   undergoes complete clinical examination, biopsy, and is scanned by computed tomography. Cats diagnosed with sublingual oral squamous cell tumors are excluded from the study. The tongue can become paralyzed as a result of such tumor, and even if the treatment kills the tumor, the animals may not be able to feed themselves. Each cat is treated repeatedly, over a longer period of time. Photographs of the tumors will be taken daily during the treatment period, and at each subsequent recheck. After treatment, each cat undergoes another computed
35   tomography scan. Computed tomography scans and thoracic radiograms are evaluated every 8 weeks thereafter. The data are evaluated for differences in survival, response and toxicity as compared to control groups. Positive

response may require evidence of tumor regression. preferably with improvement of quality of life and/or increased life span.

In addition, other spontaneous animal tumors. such as fibrosarcoma, adenocarcinoma. lymphoma, chrondroma. leiomyosarcoma of dogs. cats. and baboons can also be tested. Of these mammary adenocarcinoma in dogs and cats is a preferred model as its appearance and behavior are very similar to those in humans. However, the use of this model is limited by the rare occurrence of this type of tumor in animals.

Example 15.   Screening Assays for Drug Candidates

Screening assays for drug candidates are designed to identify compounds that bind or complex with the polypeptides encoded by the genes identified herein. or otherwise interfere with the interaction of the encoded polypeptides with other cellular proteins. Such screening assays will include assays amenable to high-throughput screening of chemical libraries. making them particularly suitable for identifying small molecule drug candidates. Small molecules contemplated include synthetic organic or inorganic compounds. including peptides, preferably soluble peptides, (poly)peptide-immunoglobulin fusions. and. in particular. antibodies including. without limitation, poly- and monoclonal antibodies and antibody fragments, single-chain antibodies. anti-idiotypic antibodies, and chimeric or humanized versions of such antibodies or fragments, as well as human antibodies and antibody fragments. The assays can be performed in a variety of formats. including protein-protein binding assays. biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art.

All assays are common in that they call for contacting the drug candidate with a polypeptide encoded by a nucleic acid identified herein under conditions and for a time sufficient to allow these two components to interact.

In binding assays, the interaction is binding and the complex formed can be isolated or detected in the reaction mixture. In a particular embodiment, the polypeptide encoded by the gene identified herein or the drug candidate is immobilized on a solid phase, e.g. on a microtiter plate, by covalent or non-covalent attachments. Non-covalent attachment generally is accomplished by coating the solid surface with a solution of the polypeptide and drying. Alternatively, an immobilized antibody, e.g. a monoclonal antibody, specific for the polypeptide to be immobilized can be used to anchor it to a solid surface. The assay is performed by adding the non-immobilized component. which may be labeled by a detectable label, to the immobilized component. e.g. the coated surface containing the anchored component. When the reaction is complete, the non-reacted components are removed, e.g. by washing, and complexes anchored on the solid surface are detected. When the originally non-immobilized component carries a detectable label, the detection of label immobilized on the surface indicates that complexing occurred. Where the originally non-immobilized component does not carry a label, complexing can be detected, for example, by using a labeled antibody specifically binding the immobilized complex.

If the candidate compound interacts with but does not bind to a particular LIV-1 polypeptide encoded by a nucleic acid sequence described herein. its interaction with that polypeptide can be assayed by methods well known for detecting protein-protein interactions. Such assays include traditional approaches, such as, cross-linking, co-immunoprecipitation, and co-purification through gradients or chromatographic columns. In addition, protein-protein interactions can be monitored by using a yeast-based genetic system described by Fields and co-workers [Fields and Song, Nature (London) 340, 245-246 (1989); Chien et al.. Proc. Natl. Acad. Sci. USA 88, 9578-

9582 (1991)] as disclosed by Chevray and Nathans [Proc. Natl. Acad. Sci. USA 89. 5789-5793 (1991)]. Many transcriptional activators, such as yeast GAL4. consist of two physically discrete modular domains. one acting as the DNA-binding domain, while the other one functioning as the transcription activation domain. The yeast expression system described in the foregoing publications (generally referred to as the "two-hybrid system") takes advantage of this property. and employs two hybrid proteins. one in which the target protein is fused to the DNA-binding domain of GAL4, and another. in which candidate activating proteins are fused to the activation domain. The expression of a GAL1-lacZ reporter gene under control of a GAL4-activated promoter depends on reconstitution of GAL4 activity via protein-protein interaction. Colonies containing interacting polypeptides are detected with a chromogenic substrate for β-galactosidase. A complete kit (MATCHMAKER™) for identifying protein-protein interactions between two specific proteins using the two-hybrid technique is commercially available from Clontech. This system can also be extended to map protein domains involved in specific protein interactions as well as to pinpoint amino acid residues that are crucial for these interactions.

Compounds that interfere with the interaction of a LIV-1-encoding gene identified herein and other intra- or extracellular components can be tested as follows: usually a reaction mixture is prepared containing the product of the amplified gene and the intra- or extracellular component under conditions and for a time allowing for the interaction and binding of the two products. To test the ability of a test compound to inhibit binding. the reaction is run in the absence and in the presence of the test compound. In addition, a placebo may be added to a third reaction mixture, to serve as positive control. The binding (complex formation) between the test compound and the intra- or extracellular component present in the mixture is monitored as described hereinabove. The formation of a complex in the control reaction(s) but not in the reaction mixture containing the test compound indicates that the test compound interferes with the interaction of the test compound and its reaction partner.

Example 16. Other Compositions and Methods for the Treatment of Tumors

The compositions useful in the treatment of tumors associated with the amplification of the genes identified herein include, without limitation, antibodies. small organic and inorganic molecules. peptides, phosphopeptides, antisense and ribozyme molecules, triple helix molecules. etc. that inhibit the expression and/or activity of the target gene product.

For example, antisense RNA and RNA molecule act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation. When antisense DNA is used. oligodeoxyribonucleotides derived from the translation initiation site, e.g. between about -10 and +10 positions of the target gene nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. Ribozymes act by sequence-specific hybridization to the complementary target RNA, followed by endonucleolytic cleavage. Specific ribozyme cleavage sites within a potential RNA target can be identified by known techniques. For further details see, e.g. Rossi, Current Biology 4, 469-471 (1994), and PCT publication No. WO 97/33551 (published September 18, 1997).

Nucleic acid molecules in triple helix formation used to inhibit transcription should be single-stranded and composed of deoxynucleotides. The base composition of these oligonucleotides is designed such that it promotes

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triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of purines or pyrimidines on one strand of a duplex. For further details see, e.g. PCT publication No. WO 97/33551, *supra*

These molecules can be identified by any or any combination of the screening assays discussed hereinabove and/or by any other screening techniques well known for those skilled in the art.

5

Deposit of Material

The following material has been deposited with the American Type Culture Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, USA (ATCC):

Material	ATCC Dep No.	Deposit Date
Plasmid:		
DNA164647 1803-1	PTA-1534	March 21, 2000
Hybridomas:		
LIV-1.2945.2G1.1C7.2F10	PTA-2961	January 23, 2001
LIV-1.2982.4A12.1E8.1C4	PTA-2962	January 23, 2001
LIV-1.2983.3G9 1D4.1D7	PTA-2963	January 23, 2001
LIV-1 2984.6D6.1H10.2C1	PTA-2964	January 23, 2001
LIV-1.2985 4F3.2D6 1D7	PTA-2960	January 23, 2001
LIV-1.2987 1D8.1C11.2B7	PTA-2959	January 23, 2001
LIV-1.2988 1A7.1F2.1H7	PTA-2965	January 23, 2001

20

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 U.S.C § 122 and the Commissioner's rules pursuant thereto (including 37 CFR § 1.14 with particular reference to 886 OG 638)

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are

functionally equivalent are within the scope of the this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and

5   described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The disclosures of all citations in the specification are expressly incorporated herein by reference.

What is claimed is:

CLAIMS

1.   An isolated nucleic acid comprising a sequence having at least 65% homology to a sequence from nucleotide 412 to and including nucleotide 477 of SEQ ID NO:3 or its complementary sequence.

2.   The isolated nucleic acid of claim 1, wherein the homology is at least 75%.

3.   The isolated nucleic acid of claim 1, wherein the homology is at least 85%.

4.   The isolated nucleic acid of claim 1, wherein the homology is at least 90%.

5.   The isolated nucleic acid of claim 1, wherein the homology is at least 96%.

6.   The isolated nucleic acid of claim 1 comprising the sequence from nucleotide 412 to and including nucleotide 477 of SEQ ID NO:3 or its complementary sequence.

7.   The isolated nucleic acid of claim 1 comprising SEQ ID NO:3.

8.   The isolated nucleic acid of claim 1 comprising a sequence from nucleotide 446 to and including nucleotide 463 of SEQ ID NO:3 or its complementary sequence.

9.   The isolated nucleic acid of claim 1, wherein the nucleic acid encodes an antigenic polypeptide.

10.   The isolated nucleic acid of claim 1, wherein the sequence encodes an extracellular domain.

11.   An isolated polypeptide comprising a sequence having at least 65% homology to SEQ ID NO:19.

12.   The isolated polypeptide of claim 11, wherein the homology is at least 75%.

13.   The isolated polypeptide of claim 11, wherein the homology is at least 85%.

14.   The isolated polypeptide of claim 11, wherein the homology is at least 90%.

15.   The isolated polypeptide of claim 11, wherein the homology is at least 96%.

16.   The isolated polypeptide of claim 11 comprising SEQ ID NO:19.

17.   The isolated polypeptide of claim 11 comprising a sequence having at least 50% homology to SEQ ID NO:17.

18.   The isolated polypeptide of claim 11 comprising SEQ ID NO:17.

19.   An isolated polypeptide comprising a sequence having at least 20% homology to SEQ ID NO:18.

20.   The isolated polypeptide of claim 11 comprising SEQ ID NO:4.

21.   The isolated polypeptide of claim 11, wherein the polypeptide is antigenic.

22.   The isolated polypeptide of claim 11, wherein the sequence comprises a portion of an extracellular domain.

23.   The isolated polypeptide of claim 11, wherein the polypeptide is aqueous soluble.

24.   An isolated polypeptide having at least 98% homology to the sequence from amino acid 1 to and including amino acid 327 of SEQ ID NO:4.

25.   An isolated polypeptide, wherein the polypeptide binds to a monoclonal antibody, wherein the antibody binds to the same epitope to which the antibody produced by hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC _____ (LIV-1.2945.2G1.1C7.2F10) binds.

26.   An isolated polypeptide, wherein the polypeptide binds to a monoclonal antibody, wherein the antibody binds to the same epitope to which the antibody produced by hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC _____ (LIV-1.2982.4A12.1E8.1C4) binds.

27.   An isolated polypeptide, wherein the polypeptide binds to a monoclonal antibody, wherein the antibody binds to the same epitope to which the antibody produced by hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC _____ (LIV-1.2983.3G9.1D4.1D7) binds.

28.   An isolated polypeptide. wherein the polypeptide binds to a monoclonal antibody. wherein the antibody
        binds to the same epitope to which the antibody produced by hybridoma cell line deposited under
        American Type Culture Collection Accession Number ATCC _____ (LIV-
        1.2984.6D6.1H10.2C1) binds.

29.   An isolated polypeptide. wherein the polypeptide binds to a monoclonal antibody. wherein the antibody
        binds to the same epitope to which the antibody produced by hybridoma cell line deposited under
        American Type Culture Collection Accession Number ATCC _____ (LIV-
        1.2985.4F3.2D6.1D7) binds.

30.   An isolated polypeptide. wherein the polypeptide binds to a monoclonal antibody. wherein the antibody
        binds to the same epitope to which the antibody produced by hybridoma cell line deposited under
        American Type Culture Collection Accession Number ATCC _____ (LIV-
        1.2987.1D8.1C11.2B7) binds.

31.   An isolated polypeptide. wherein the polypeptide binds to a monoclonal antibody. wherein the antibody
        binds to the same epitope to which the antibody produced by hybridoma cell line deposited under
        American Type Culture Collection Accession Number ATCC _____ (LIV-
        1.2988.1A7.1F2.1H7) binds.

32.   A monoclonal antibody that binds a polypeptide, wherein the polypeptide comprises a sequence that is
        at least 80% homologous to the sequence from amino acid 1 to and including amino acid 327 of SEQ
        ID NO:4.

33.   The monoclonal antibody of claim 32, wherein the homology is at least 98%.

34.   A monoclonal antibody that binds a polypeptide, wherein the polypeptide comprises a sequence that is
        at least 65% homologous to SEQ ID NO:19.

35.   The monoclonal antibody of claim 32, wherein the homology is at least 75%.

36.   The monoclonal antibody of claim 32, wherein the homology is at least 85%.

37.   The monoclonal antibody of claim 32, wherein the homology is at least 90%.

38.   The monoclonal antibody of claim 32, wherein the homology is at least 96%.

39.   The monoclonal antibody of claim 32, wherein the polypeptide comprises SEQ ID NO:19.

40.   The monoclonal antibody of claim 32, wherein the polypeptide comprises SEQ ID NO:17.

41.   The monoclonal antibody of claim 32, wherein the antibody is a LIV-1-164647 antagonist.

42.   The monoclonal antibody of claim 32, wherein the antibody inhibits proliferation of tumor cells.

43.   The monoclonal antibody of claim 42, wherein the tumors cells are selected from the group consisting of breast, lung, prostate, colon, ovary, uterus, kidney, gastric, and salivary gland carcinomas, or other tumor cell types expressing the LIV-1-164647 protein.

44.   The monoclonal antibody as in claim 42, wherein the antibody inhibits *in vitro* proliferation of tumor cells that overexpress LIV-1.

45.   The monoclonal antibody as in claim 32, which antibody is a murine monoclonal antibody.

46.   The monoclonal antibody as in claim 32, which antibody is a murine-human hybrid antibody.

47.   The monoclonal antibody as in claim 32, wherein the antibody competes for binding to the ligand binding site of a ligand of the LIV-1 protein.

48.   The monoclonal antibody as in claim 32, wherein the antibody reduces expression LIV-1.

49.   The monoclonal antibody as in claim 32, wherein the antibody activates complement.

50.   The monoclonal antibody as in claim 32, wherein the antibody mediates antibody dependent cellular cytotoxicity.

51.   A monoclonal antibody that binds to an epitope, wherein the epitope comprises an amino acid sequence having at least 65% homology to SEQ ID NO:19.

52.   The monoclonal antibody of claim 49, wherein the epitope comprises an amino acid sequence having at least 80% homology to SEQ ID NO:17.

53.   The monoclonal antibody of claim 52, wherein the epitope comprises SEQ ID NO:17.

54.   A monoclonal antibody, wherein the antibody binds to the same epitope as the epitope to which the
        monoclonal antibody produced by a hybridoma cell line binds, wherein the hybridoma cell line is
        selected from the group consisting of hybridoma cell lines deposited under American Type Culture
        Collection Accession Numbers ATCC_____ (LIV-1.2945.2G1.1C7.2F10); ATCC _____ (LIV-
        1.2982.4A12.1E8.1C4); ATCC _____ (LIV-1.2983.3G9.1D4.1D7); ATCC _____ (LIV-
        1.2984.6D6.1H10.2C1); ATCC _____ (LIV-1.2985.4F3.2D6.1D7); ATCC _____ (LIV-
        1.2987.1D8.1C11.2B7); ATCC _____ (LIV-1.2988.1A7.1F2.1H7).

55.   An immunotoxin which is a conjugate of a cytotoxic moiety and the monoclonal antibody of claim 32.

56.   A hybridoma producing the monoclonal antibody of claim 32.

57.   A hybridoma selected from the group consisting of ATCC_____ (LIV-1.2945.2G1.1C7.2F10);
        ATCC _____ (LIV-1.2982.4A12.1E8.1C4); ATCC _____ (LIV-1.2983.3G9.1D4.1D7); ATCC
        _____ (LIV-1.2984.6D6.1H10.2C1); ATCC _____ (LIV-1.2985.4F3.2D6.1D7); ATCC _____ (LIV-
        1.2987.1D8.1C11.2B7); ATCC _____ (LIV-1.2988.1A7.1F2.1H7).

58.   An assay for detecting a tumor, the method comprising:
                (a) exposing a sample from a mammal to an antibody that specifically binds a polypeptide
        comprising a sequence having at least 80% homologous to a sequence from amino acid 1 to and
        including amino acid 327 of SEQ ID NO:4; and
                (b) determining the extent of binding to a polypeptide in the sample.

59.   The assay of claim 58, wherein the polypeptide in the sample is on a cell from the mammal.

60.   The assay of claim 58, wherein the sample is body fluid from the mammal and the polypeptide is a
        soluble polypeptide.

61.   The assay as in claim 58, wherein the antibody is a monoclonal antibody.

62.   The assay as in claim 58, wherein the assay is performed *in vitro* and the assay in an ELISA assay.

63.   The assay as in claim 59, wherein the cell is a plurality of cells which remain within the body of a
        mammal, the antibody is a plurality of antibodies which are tagged with a radioactive isotope and
        administered to the mammal, and the extent of binding of the antibodies to the cells is observed by
        external scanning for radioactivity.

64.   The assay as in claim 58, wherein the assay further comprises anti-ErbB2 antibodies tagged with a
        second radioactive isotope and administered to the mammal, and the extent of binding of the antibodies
        to the cells by the tagged anti-ErbB2 antibodies to the cells is observed by external scanning for
        radioactivity.

65.   A method of inhibiting the growth of tumor cells, the method comprising:
                administering to a mammal a therapeutically effective amount of an antibody capable of
        binding to an amino acid sequence having at least 80% homology to a sequence from amino acid 1 to
        and including amino acid 327 of SEQ ID NO:4, which antibody inhibits proliferation of LIV-1-
        164647-expressing cells.

66.   The method of claim 65, wherein the antibodies activate complement.

67.   The method of claim 65, wherein the antibodies mediate antibody dependent cellular cytotoxicity.

68.   The method of claim 65, wherein the antibodies are monoclonal antibodies.

69.   The method of claim 65, wherein the tumor cells comprise a carcinoma selected from breast, lung,
        prostate, colon, ovary, uterus, kidney, gastric, and salivary gland carcinomas, or other tumor cell types
        expressing the LIV-1-164647 protein.

70.   The method of claim 69, wherein the tumor cells are breast tissue cells.

71.   The method of claim 69, wherein the mammal is a human.

72.   The method of claim 65, wherein the method further comprises administering a therapeutically
        effective amount of a cytotoxic factor.

73.   The method of claim 72, wherein the antibodies and cytotoxic factor are administered simultaneously.

74.   The method of claim 72, wherein the antibody is administered to the patient before the cytoxic agent.

75.   The method of claim 72, wherein the cytotoxic agent is administered before the antibody.

76.   The method of claim 72, wherein the cytotoxic agent is conjugated to the antibody.

77.   A composition comprising an antibody that binds specifically to an amino acid sequence having at least 80% homologous to a sequence from amino acid 1 to and including amino acid 327 of SEQ ID NO:4. and a pharmaceutically acceptable carrier.

78.   A composition comprising an antibody and a pharmaceutically acceptable carrier, wherein the antibody is a monoclonal antibody produced by a hybridoma cell line selected from the group consisting of ATCC_____ (LIV-1.2945.2G1.1C7.2F10); ATCC _____ (LIV-1.2982.4A12.1E8.1C4); ATCC _____ (LIV-1.2983.3G9.1D4.1D7); ATCC _____ (LIV-1.2984.6D6.1H10.2C1); ATCC _____ (LIV-1.2985.4F3.2D6.1D7); ATCC _____ (LIV-1.2987.1D8.1C11.2B7); ATCC _____ (LIV-1.2988.1A7.1F2.1H7).

79.   The composition of claim 77, further comprising a cytotoxic factor.

80.   A method of diagnosing tumor in a mammal, comprising detecting the level of expression of a nucleic acid sequence comprising a sequence having at least 65% homology to a sequence from nucleotide 412 to and including nucleotide 477 of SEQ ID NO:3 or its complementary sequence in a test sample obtained from the mammal, and in a control sample, wherein a higher expression level in the test sample indicates the presence of tumor in the mammal from which the test sample was obtained.

81.   A method of diagnosing tumor in a mammal, comprising:
        (a) contacting an antibody with a test sample obtained from the mammal; and
        (b) detecting the formation of a complex between the antibody and a polypeptide of the test sample, wherein the antibody binds the polypeptide, the polypeptide comprising an amino acid sequence having at least 80% homology to the sequence from amino acid 1 to and including amino acid 327 of SEQ ID NO:4.

82.   The method of claim 80, wherein said test sample is obtained from an individual suspected to have neoplastic cell growth or proliferation.

83.   The method of claim 81, wherein the test sample is obtained from an individual suspected to have neoplastic cell growth or proliferation.

84.   An article of manufacture comprising:
        a container; and
        a composition of claim 77 contained within the container.

85.   An article of manufacture comprising:

a container; and

a composition of claim 78 contained with the container.

86.   An article of manufacture, comprising:

a container;

a label on the container; and

the composition of claim 77, wherein the composition is effective for inhibiting the growth of tumor cells, and the label on the container indicates that the composition can be used for treating conditions characterized by overexpression of a LIV-1-164647 polypeptide.

87.   An article of manufacture, comprising:

a container;

a label on the container; and

the composition of claim 78, wherein the composition is effective for inhibiting the growth of tumor cells, and the label on the container indicates that the composition can be used for treating conditions characterized by overexpression of a LIV-1-164647 polypeptide.

88.   A method for identifying a compound capable of inhibiting the expression or activity of a LIV-1-164647 polypeptide, comprising contacting a candidate compound with a polypeptide comprising a sequence having at least 65% homology to SEQ ID NO:19.

89.   The method of claim 88 wherein the candidate compound or the polypeptide is immobilized on a solid support.

90.   The method of claim 89 wherein the non-immobilized component carries a detectable label.

91.   A host cell comprising a nucleic acid sequence having at least 65% nucleic acid sequence homology to the sequence from nucleotide 412 to and including nucleotide 477 of SEQ ID NO:3 or its complementary sequence.

92.   The host cell of claim 91, wherein the cell is a Chinese hamster ovary (CHO) cell.

93.   The host cell of claim 91, wherein the cell is an *E. coli*.

94.   The host cell of claim 91, wherein the cell is a yeast cell.

95.   The host cell of claim 91, wherein the cell is an insect cell.

96.   The antibody of claim 32, wherein the antibody is a chimeric antibody.

97.   The antibody of claim 32, wherein the antibody is a humanized antibody.

98.   The antibody of claim 32, wherein the antibody is a human antibody.

99.   The antibody of claim 32, wherein the antibody is an antibody fragment selected from the gropu
        consisting of Fab, Fab', F(ab')₂, Fv fragments, diabodies, single chain antibodies, and multispecific
        antibodies.

100.   A host cell which produces the antibody of claim 32, wherein the host cell is selected from the group
        consisting of a Chinese Hamster Ovary (CHO) cell, *E. coli*, yeast cell, and insect cell.

CTCGTGCCGAATTCGGCACGAGACCGCGTGTTCGCGCCTGGTAGAGATTTCTCGAAGACA

CCAGTGGGCCCGTGTGGAACCAAACCTGCGCGCGTGGCCGGGCCGTGGGACAACGAGGCC

GCGGAGACGAAGGCGCAATGGCGAGGAAGTTATCTGTAATCTTGATCCTGACCTTTGCCC

TCTCTGTCACAAATCCCCTTCATGAACTAAAAGCAGCTGCTTTCCCCCAGACCACTGAGA

AAATTAGTCCGAATTGGGAATCTGGCATTAATGTTGACTTGGCAATTTCCACACGGCAAT

ATCATCTACAACAGCTTTTCTACCGCTATGGAGAAAATAATTCTTTGTCAGTTGAAGGGT

TCAGAAAATTACTTCAAAATATAGGCATAGATAAGATTAAAAGAATCCATATACACCATG

ACCACGACCATCACTCAGACCACGAGCATCACTCAGACCATGAGCGTCACTCAGACCATG

AGCATCACTCAGACCACGAGCATCACTCTGACCATAATCATGCTGCTTCTGGTAAAAATA

AGCGAAAAGCTCTTTGCCCAGACCATGACTCAGATAGTTCAGGTAAAGATCCTAGAAACA

GCCAGGGGAAAGGAGCTCACCGACCAGAACATGCCAGTGGTAGAAGGAATGTCAAGGACA

GTGTTAGTGCTAGTGAAGTGACCTCAACTGTGTACAACACTGTCTCTGAAGGAACTCACT

TTCTAGAGACAATAGAGACTCCAAGACCTGGAAAACTCTTCCCCAAAGATGTAAGCAGCT

CCACTCCACCCAGTGTCACATCAAAGAGCCGGGTGAGCCGGCTGGCTGGTAGGAAAACAA

ATGAATCTGTGAGTGAGCCCCGAAAAGGCTTTATGTATTCCAGAAACACAAATGAAAATC

CTCAGGAGTGTTTCAATGCATCAAAGCTACTGACATCTCATGGCATGGGCATCCAGGTTC

CGCTGAATGCAACAGAGTTCAACTATCTCTGTCCAGCCATCATCAACCAAATTGATGCTA

GATCTTGTCTGATTCATACAAGTGAAAAGAAGGCTGAAATCCCTCCAAAGACCTATTCAT

TACAAATAGCCTGGGTTGGTGGTTTTATAGCCATTTCCATCATCAGTTTCCTGTCTCTGC

TGGGGGTTATCTTAGTGCCTCTCATGAATCGGGTGTTTTTCAAATTTCTCCTGAGTTTCC

TTGTGGCACTGGCCGTTGGGACTTTGAGTGGTGATGCTTTTTTACACCTTCTTCCACATT

CTCATGCAAGTCACCACCATAGTCATAGCCATGAAGAACCAGCAATGGAAATGAAAAGAG

GACCACTTTTCAGTCATCTGTCTTCTCAAAACATAGAAGAAAGTGCCTATTTTGATTCCA

CGTGGAAGGGTCTAACAGCTCTAGGAGGCCTGTATTTCATGTTTCTTGTTGAACATGTCC

TCACATTGATCAAACAATTTAAAGATAAGAAGAAAAAGAATCAGAAGAAACCTGAAAATG

ATGATGATGTGGAGATTAAGAAGCAGTTGTCCAAGTATGAATCTCAACTTTCAACAAATG

AGGAGAAAGTAGATACAGATGATCGAACTGAAGGCTATTTACGAGCAGACTCACAAGAGC

CCTCCCACTTTGATTCTCAGCAGCCTGCAGTCTTGGAAGAAGAAGAGGTCATGATAGCTC

ATGCTCATCCACAGGAAGTCTACAATGAATATGTACCCAGAGGGTGCAAGAATAAATGCC

ATTCACATTTCCACGATACACTCGGCCAGTCAGACGATCTCATTCACCACCATCATGACT

ACCATCATATTCTCCATCATCACCACCACCAAAACCACCATCCTCACAGTCACAGCCAGC

GCTACTCTCGGGAGGAGCTGAAAGATGCCGGCGTCGCCACTTTGGCCTGGATGGTGATAA

Fig. 1A-1

TGGGTGATGGCCTGCACAATTTCAGCGATGGCCTAGCAATTGGTGCTGCTTTTACTGAAG

GCTTATCAAGTGGTTTAAGTACTTCTGTTGCTGTGTTCTGTCATGAGTTGCCTCATGAAT

TAGGTGACTTTGCTGTTCTACTAAAGGCTGGCATGACCGTTAAGCAGGCTGTCCTTTATA

ATGCATTGTCAGCCATGCTGGCGTATCTTGGAATGGCAACAGGAATTTTCATTGGTCATT

ATGCTGAAAATGTTTCTATGTGGATATTTGCACTTACTGCTGGCTTATTCATGTATGTTG

CTCTGGTTGATATGGTACCTGAAATGCTGCACAATGATGCTAGTGACCATGGATGTAGCC

GCTGGGGGTATTTCTTTTTACAGAATGCTGGGATGCTTTTGGGTTTTGGAATTATGTTAC

TTATTCCATATTTGAACATAAAATCGTGTTCGTATAAATTTCTAGTTAAGGTTTAAATGC

TAGAGTAGCTTAAAAAGTTGTCATAGTTTCAGTAGGTCATAGGGAGATGAGTTTGTATGC

TGTACTATGCAGCGTTTAAAGTTAGTGGGTTTTGTGATTTTTGTATTGAATATTGCTGTC

TGTTACAAAGTCAGTTAAAGGTACGTTTTAATATTTAAGTTATTCTATCTTGGAGATAAA

ATCTGTATGTGCAATTCACCGGTATTACCAGTTTATTATGTAAACAAGAGATTTGGCATG

ACATGTTCTGTATGTTTCAGGGAAAAATGTCTTTAATGCTTTTTCAAGAACTAACACAGT

TATTCCTATACTGGATTTTAGGTCTCTGAAGAACTGCTGGTGTTAGGAATAAGAATGTG

CATGAAGCCTAAAATACCAAGAAAGCTTATACTGAATTTAAGCAAAGAAATAAAGGAGAA

AAGAGAAGAATCTGAGAATTGGGGAGGCATAGATTCTTATAAAAATCACAAAATTTGTTG

TAAATTAGAGGGGAGAAATTTAGAATTAAGTATAAAAAGGCAGAATTAGTATAGAGTACA

TTCATTAAACATTTTTGTCAGGATTATTTCCCGTAAAAACGTAGTGAGCACTCTCATATA

CTAATTAGTGTACATTTAACTTTGTATAATACAGAAATCTAAATATATTTAATGAATTCA

AGCAATATACACTTGACCAAGAAATTGGAATTTCAAAATGTTCGTGCGGGTTATATACCA

GATGAGTACAGTGAGTAGTTTATGTATCACCAGACTGGGTTATTGCCAAGTTATATATCA

CCAAAAGCTGTATGACTGGATGTTCTGGTTACCTGGTTTACAAAATTATCAGAGTAGTAA

AACTTTGATATATATGAGGATATTAAAACTACACTAAGTATCATTTGATTCGATTCAGAA

AGTACTTTGATATCTCTCAGTGCTTCAGTGCTATCATTGTGAGCAATTGTCTTTATATAC

GGTACTGTAGCCATACTAGGCCTGTCTGTGGCATTCTCTAGATGTTTCTTTTTTACACAA

TAAATTCCTTATATCAGCTTGAAAAAAAAAAAAAAAAAAAAAA   (SEQ ID NO:1)

Fig. 1A-2

MARKLSVILILTFALSVTNPLHELKAAAFPQTTEKISPNWESGINVDLAISTRQYHLQQL
FYRYGENNSLSVEGFRKLLQNIGIDKIKRIHIHHDHDHHSDHEHHSDHERHSDHEHHSDH
EHHSDHNHAASGKNKRKALCPDHDSDSSGKDPRNSQGKGAHRPEHASGRRNVKDSVSASE
VTSTVYNTVSEGTHFLETIETPRPGKLFPKDVSSSTPPSVTSKSRVSRLAGRKTNESVSE
PRKGFMYSRNTNENPQECFNASKLLTSHGMGIQVPLNATEFNYLCPAIINQIDARSCLIH
TSEKKAEIPPKTYSLQIAWVGGFIAISIISFLSLLGVILVPLMNRVFFKFLLSFLVALAV
GTLSGDAFLHLLPHSHASHHHSHSHEEPAMEMKRGPLFSHLSSQNIEESAYFDSTWKGLT
ALGGLYFMFLVEHVLTLIKQFKDKKKKNQKKPENDDDVEIKKQLSKYESQLSTNEEKVDT
DDRTEGYLRADSQEPSHFDSQQPAVLEEEEVMIAHAHPQEVYNEYVPRGCKNKCHSHFHD
TLGQSDDLIHHHHDYHHILHHHHHHQNHHPHSHSQRYSREELKDAGVATLAWMVIMGDGLH
NFSDGLAIGAAFTEGLSSGLSTSVAVFCHELPHELGDFAVLLKAGMTVKQAVLYNALSAM
LAYLGMATGIFIGHYAENVSMWIFALTAGLFMYVALVDMVPEMLHNDASDHGCSRWGYFF
LQNAGMLLGFGIMLLIPYLNIKSCSYKFLVKV   (SEQ ID NO:2)

Fig. 1B

TTTTTTTTTGATATAAGGAATTTATTGTGTAAAAAAGAAACATCTAGAGAATGCCACAGA

CAGGCCTAGTATGGCTACAGTACCGTATATAAAAGACAATTGCTCACAATGATAGCACTG

AAGCACTGAGAGATATCAAAGTACTTTCTGAATCGAATCAAATGATACTTAGTGTAGTTT

TAATATCCTCATATATATCAAAGTTTTACTACTCTGATAATTTTGTAAACCAGGGTAACC

AGGAXCATCCAGTCATACAGCTTTTGGGTGATATATAACTTGGGCAATAACCCAGTCTGG

GTGATACXTAAAXCTACTCACTGTACTCATCTGGGTATATACCCGCACGGAXCATTTTGG

AAATTCCCAATTTCTTGGGTCAGGTGATATA   (SEQ ID NO:5)

Fig. 1C

```
                10        20        30        40        50
ss.DNA164647  CCGGCCGTGTGGAACCAAACCTGCGCGCGTGGCCGGGCCGTGGGACAACGAGGCC


                60        70        80        90        100       110
ss.DNA164647  GCGGAGACGAAGGCGCAATGGCGAGGAAGTTATCTGTAATCTTGATCCTGACCTTTGCCC


                120       130       140       150       160       170
ss.DNA164647  TCTCTGTCACAAATCCCCTTCATGAACTAAAAGCAGCTGCTTTCCCCCAGACCACTGAGA


                180       190       200       210       220       230
ss.DNA164647  AAATTAGTCCGAATTGGGAATCTGGCATTAATGTTGACTTGGCAATTTCCACACGGCAAT


                240       250       260       270       280       290
ss.DNA164647  ATCATCTACAACAGCTTTTCTACCGCTATGGAGAAAATAATTCTTTGTCAGTTGAAGGGT


                300       310       320       330       340       350
ss.DNA164647  TCAGAAAATTACTTCAAAATATAGGCATAGATAAGATTAAAAGAATCCATATACACCATG


                360       370       380       390       400       410
ss.DNA164647  ACCACGACCATCACTCAGACCACGAGCATCACTCAGACCATGAGCGTCACTCAGACCATG


                420       430       440       450       460       470
ss.DNA164647  AGCATCACTCAGACCACGAGCATCACTCTGACCATGATCATCACTCCCACCATAATCATG


                480       490       500       510       520       530
ss.DNA164647  CTGCTTCTGGTAAAAATAAGCGAAAAGCTCTTTGCCCAGACCATGACTCAGATAGTTCAG


                540       550       560       570       580       590
ss.DNA164647  GTAAAGATCCTAGAAACAGCCAGGGGAAAGGAGCTCACCGACCAGAACATGCCAGTGGTA


                600       610       620       630       640       650
ss.DNA164647  GAAGGAATGTCAAGGACAGTGTTAGTGCTAGTGAAGTGACCTCAACTGTGTACAACACTG
```

Fig. 2A-1

```
            660        670        680        690        700        710
ss.DNA164647  TCTCTGAAGGAACTCACTTTCTAGAGACAATAGAGACTCCAAGACCTGGAAAACTCTTCC


            720        730        740        750        760        770
ss.DNA164647  CCAAAGATGTAAGCAGCTCCACTCCACCCAGTGTCACATCAAAGAGCCGGGTGAGCCGGC


            780        790        800        810        820        830
ss.DNA164647  TGGCTGGTAGGAAAACAAATGAATCTGTGAGTGAGCCCCGAAAAGGCTTTATGTATTCCA


            840        850        860        870        880        890
ss.DNA164647  GAAACACAAATGAAAATCCTCAGGAGTGTTTCAATGCATCAAAGCTACTGACATCTCATG


            900        910        920        930        940        950
ss.DNA164647  GCATGGGCATCCAGGTTCCGCTGAATGCAACAGAGTTCAACTATCTCTGTCCAGCCATCA


            960        970        980        990        1000       1010
ss.DNA164647  TCAACCAAATTGATGCTAGATCTTGTCTGATTCATACAAGTGAAAAGAAGGCTGAAATCC


            1020       1030       1040       1050       1060       1070
ss.DNA164647  CTCCAAAGACCTATTCATTACAAATAGCCTGGGTTGGTGGTTTTATAGCCATTTCCATCA


            1080       1090       1100       1110       1120       1130
ss.DNA164647  TCAGTTTCCTGTCTCTGCTGGGGGGTTATCTTAGTGCCTCTCATGAATCGGGTGTTTTTCA


            1140       1150       1160       1170       1180       1190
ss.DNA164647  AATTTCTCCTGAGTTTCCTTGTGGCACTGGCCGTTGGGACTTTGAGTGGTGATGCTTTTT


            1200       1210       1220       1230       1240       1250
ss.DNA164647  TACACCTTCTTCCACATTCTCATGCAAGTCACCACCATAGTCATAGCCATGAAGAACCAG


            1260       1270       1280       1290       1300       1310
ss.DNA164647  CAATGGAAATGAAAAGAGGACCACTTTTCAGTCATCTGTCTTCTCAAAACATAGAAGAAA
```

Fig. 2A-2

```
                1320      1330      1340      1350      1360      1370
ss.DNA164647    GTGCCTATTTTGATTCCACGTGGAAGGGTCTAACAGCTCTAGGAGGCCTGTATTTCATGT
```

```
                1380      1390      1400      1410      1420      1430
ss.DNA164647    TTCTTGTTGAACATGTCCTCACATTGATCAAACAATTTAAAGATAAGAAGAAAAAGAATC
```

```
                1440      1450      1460      1470      1480      1490
ss.DNA164647    AGAAGAAACCTGAAAATGATGATGATGTGGAGATTAAGAAGCAGTTGTCCAAGTATGAAT
```

```
                1500      1510      1520      1530      1540      1550
ss.DNA164647    CTCAACTTTCAACAAATGAGGAGAAAGTAGATACAGATGATCGAACTGAAGGCTATTTAC
```

```
                1560      1570      1580      1590      1600      1610
ss.DNA164647    GAGCAGACTCACAAGAGCCCTCCCACTTTGATTCTCAGCAGCCTGCAGTCTTGGAAGAAG
```

```
                1620      1630      1640      1650      1660      1670
ss.DNA164647    AAGAGGTCATGATAGCTCATGCTCATCCACAGGAAGTCTACAATGAATATGTACCCAGAG
```

```
                1680      1690      1700      1710      1720      1730
ss.DNA164647    GGTGCAAGAATAAATGCCATTCACATTTCCACGATACACTCGGCCAGTCAGACGATCTCA
```

```
                1740      1750      1760      1770      1780      1790
ss.DNA164647    TTCACCACCATCATGACTACCATCATATTCTCCATCATCACCACCACCAAAACCACCATC
```

```
                1800      1810      1820      1830      1840      1850
ss.DNA164647    CTCACAGTCACAGCCAGCGCTACTCTCGGGAGGAGCTGAAAGATGCCGGCGTCGCCACTT
```

```
                1860      1870      1880      1890      1900      1910
ss.DNA164647    TGGCCTGGATGGTGATAATGGGTGATGGCCTGCACAATTTCAGCGATGGCCTAGCAATTG
```

```
                1920      1930      1940      1950      1960      1970
ss.DNA164647    GTGCTGCTTTTACTGAAGGCTTATCAAGTGGTTTAAGTACTTCTGTTGCTGTGTTCTGTC
```

Fig. 2A-3

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                1980      1990      2000      2010      2020      2030
ss.DNA164647    ATGAGTTGCCTCATGAATTAGGTGACTTTGCTGTTCTACTAAAGGCTGACATGACCGTTA

                2040      2050      2060      2070      2080      2090
ss.DNA164647    AGCAGGCTGTCCTTTATAATGCATTGTCAGCCATGCTGGCGTATCTTGGAATGGCAACAG

                2100      2110      2120      2130      2140      2150
ss.DNA164647    GAATTTTCATTGGTCATTATGCTGAAAATGTTTCTATGTGGATATTTGCACTTACTGCTG

                2160      2170      2180      2190      2200      2210
ss.DNA164647    GCTTATTCATGTATGTTGCTCTGGTTGATATGGTACCTGAAATGCTGCACAATGATGCTA

                2220      2230      2240      2250      2260      2270
ss.DNA164647    GTGACCATGGATGTAGCCGCTGGGGGTATTTCTTTTTACAGAATGCTGGGATGCTTTTGG

                2280      2290      2300      2310      2320      2330
ss.DNA164647    GTTTTGGAATTATGTTACTTATTTCCATATTTGAACATAAAATCGTGTTTCGTATAAATT

                2340      2350      2360      2370      2380      2390
ss.DNA164647    TCTAGTTAAGGTTTAAATGCTAGAGTAGCTTAAAAAGTTGTCATAGTTTCAGTAGGTCAT

                2400      2410      2420      2430      2440      2450
ss.DNA164647    AGGGAGATGAGTTTGTATGCTGTACTATGCAGCGTTTAAAGTTAGTGGGTTTTGTGATTT

                2460      2470      2480      2490      2500      2510
ss.DNA164647    TTGTATTGAATATTGCTGTCTGTTACAAAGTCAGTTAAAGGTACGTTTTAATATTTAAGT

                2520      2530      2540      2550      2560      2570
ss.DNA164647    TATTCTATCTTGGAGATAAAATCTGTATGTGCAATTCACCGGTATTACCAGTTTATTATG

                2580      2590      2600      2610      2620      2630
ss.DNA164647    TAAACAAGAGATTTGGCATGACATGTTCTGTATGTTTCAGGGAAAAATGTCTTTAATGCT
```

Fig. 2A-4

```
          2640      2650      2660      2670      2680      2690
ss.DNA164647   TTTTCAAGAACTAACACAGTTATTCCTATACTGGATTTTAGGTCTCTGAAGAACTGCTGG


          2700      2710      2720      2730      2740      2750
ss.DNA164647   TGTTTAGGAATAAGAATGTGCATGAAGCCTAAAATACCAAGAAAGCTTATACTGAATTTA


          2760      2770
ss.DNA164647   AGCAAAAAAAAAAAAAAAAAAAA              (SEQ ID NO:3)
```

Fig. 2A-5

```
Met Ala Arg Lys Leu Ser Val Ile Leu Ile Leu Thr Phe Ala Leu
  1           5             10             15

Ser Val Thr Asn Pro Leu His Glu Leu Lys Ala Ala Ala Phe Pro
             20             25             30

Gln Thr Thr Glu Lys Ile Ser Pro Asn Trp Glu Ser Gly Ile Asn
             35             40             45

Val Asp Leu Ala Ile Ser Thr Arg Gln Tyr His Leu Gln Gln Leu
             50             55             60

Phe Tyr Arg Tyr Gly Glu Asn Asn Ser Leu Ser Val Glu Gly Phe
             65             70             75

Arg Lys Leu Leu Gln Asn Ile Gly Ile Asp Lys Ile Lys Arg Ile
             80             85             90

His Ile His His Asp His Asp His His Ser Asp His Glu His His
             95            100            105

Ser Asp His Glu Arg His Ser Asp His Glu His His Ser Asp His
            110            115            120

Glu His His Ser Asp His Asp His His Ser His His Asn His Ala
            125            130            135

Ala Ser Gly Lys Asn Lys Arg Lys Ala Leu Cys Pro Asp His Asp
            140            145            150

Ser Asp Ser Ser Gly Lys Asp Pro Arg Asn Ser Gln Gly Lys Gly
            155            160            165

Ala His Arg Pro Glu His Ala Ser Gly Arg Arg Asn Val Lys Asp
            170            175            180

Ser Val Ser Ala Ser Glu Val Thr Ser Thr Val Tyr Asn Thr Val
            185            190            195

Ser Glu Gly Thr His Phe Leu Glu Thr Ile Glu Thr Pro Arg Pro
            200            205            210

Gly Lys Leu Phe Pro Lys Asp Val Ser Ser Ser Thr Pro Pro Ser
            215            220            225

Val Thr Ser Lys Ser Arg Val Ser Arg Leu Ala Gly Arg Lys Thr
            230            235            240

Asn Glu Ser Val Ser Glu Pro Arg Lys Gly Phe Met Tyr Ser Arg
            245            250            255

Asn Thr Asn Glu Asn Pro Gln Glu Cys Phe Asn Ala Ser Lys Leu
            260            265            270

Leu Thr Ser His Gly Met Gly Ile Gln Val Pro Leu Asn Ala Thr
            275            280            285

Glu Phe Asn Tyr Leu Cys Pro Ala Ile Ile Asn Gln Ile Asp Ala
            290            295            300
```

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Fig. 2B-1

```
Arg Ser Cys Leu Ile His Thr Ser Glu Lys Lys Ala Glu Ile Pro
             305             310             315

Pro Lys Thr Tyr Ser Leu Gln Ile Ala Trp Val Gly Gly Phe Ile
             320             325             330

Ala Ile Ser Ile Ile Ser Phe Leu Ser Leu Leu Gly Val Ile Leu
             335             340             345

Val Pro Leu Met Asn Arg Val Phe Phe Lys Phe Leu Leu Ser Phe
             350             355             360

Leu Val Ala Leu Ala Val Gly Thr Leu Ser Gly Asp Ala Phe Leu
             365             370             375

His Leu Leu Pro His Ser His Ala Ser His His His Ser His Ser
             380             385             390

His Glu Glu Pro Ala Met Glu Met Lys Arg Gly Pro Leu Phe Ser
             395             400             405

His Leu Ser Ser Gln Asn Ile Glu Glu Ser Ala Tyr Phe Asp Ser
             410             415             420

Thr Trp Lys Gly Leu Thr Ala Leu Gly Gly Leu Tyr Phe Met Phe
             425             430             435

Leu Val Glu His Val Leu Thr Leu Ile Lys Gln Phe Lys Asp Lys
             440             445             450

Lys Lys Lys Asn Gln Lys Lys Pro Glu Asn Asp Asp Asp Val Glu
             455             460             465

Ile Lys Lys Gln Leu Ser Lys Tyr Glu Ser Gln Leu Ser Thr Asn
             470             475             480

Glu Glu Lys Val Asp Thr Asp Asp Arg Thr Glu Gly Tyr Leu Arg
             485             490             495

Ala Asp Ser Gln Glu Pro Ser His Phe Asp Ser Gln Gln Pro Ala
             500             505             510

Val Leu Glu Glu Glu Glu Val Met Ile Ala His Ala His Pro Gln
             515             520             525

Glu Val Tyr Asn Glu Tyr Val Pro Arg Gly Cys Lys Asn Lys Cys
             530             535             540

His Ser His Phe His Asp Thr Leu Gly Gln Ser Asp Asp Leu Ile
             545             550             555

His His His His Asp Tyr His His Ile Leu His His His His His
             560             565             570

Gln Asn His His Pro His Ser His Ser Gln Arg Tyr Ser Arg Glu
             575             580             585

Glu Leu Lys Asp Ala Gly Val Ala Thr Leu Ala Trp Met Val Ile
             590             595             600
```

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Fig. 2B-2

```
Met Gly Asp Gly Leu His Asn Phe Ser Asp Gly Leu Ala Ile Gly
                  605             610             615

Ala Ala Phe Thr Glu Gly Leu Ser Ser Gly Leu Ser Thr Ser Val
                  620             625             630

Ala Val Phe Cys His Glu Leu Pro His Glu Leu Gly Asp Phe Ala
                  635             640             645

Val Leu Leu Lys Ala Asp Met Thr Val Lys Gln Ala Val Leu Tyr
                  650             655             660

Asn Ala Leu Ser Ala Met Leu Ala Tyr Leu Gly Met Ala Thr Gly
                  665             670             675

Ile Phe Ile Gly His Tyr Ala Glu Asn Val Ser Met Trp Ile Phe
                  680             685             690

Ala Leu Thr Ala Gly Leu Phe Met Tyr Val Ala Leu Val Asp Met
                  695             700             705

Val Pro Glu Met Leu His Asn Asp Ala Ser Asp His Gly Cys Ser
                  710             715             720

Arg Trp Gly Tyr Phe Phe Leu Gln Asn Ala Gly Met Leu Leu Gly
                  725             730             735

Phe Gly Ile Met Leu Leu Ile Ser Ile Phe Glu His Lys Ile Val
                  740             745             750

Phe Arg Ile Asn Phe   (SEQ ID NO:4)
                  755
```

Fig. 2B-3

ss.u41060   CTCGTGCCGAATTCGGCACGAGACCGCGTGTTCGCGCCTGGTAGAGATTTCTCGAAGACA
                          10   20   30   40   50   60

                          10   20   30   40   50
ss.DNA164647   CCGGCCGTGTGGAACCAAACCTGCGCGCGTGGCCGGGCCGTGGGACAACGAGGCC
                    * ***
ss.u41060   CCAGTGGGCCCGTGTGGAACCAAACCTGCGCGCGTGGCCGGGCCGTGGGACAACGAGGCC
                          70   80   90   100   110   120

                          60   70   80   90   100   110
ss.DNA164647   GCGGAGACGAAGGCGCAATGGCGAGGAAGTTATCTGTAATCTTGATCCTGACCTTTGCCC
                    ****
ss.u41060   GCGGAGACGAAGGCGCAATGGCGAGGAAGTTATCTGTAATCTTGATCCTGACCTTTGCCC
                         130   140   150   160   170   180

                         120   130   140   150   160   170
ss.DNA164647   TCTCTGTCACAAATCCCCTTCATGAACTAAAAGCAGCTGCTTTCCCCCAGACCACTGAGA
                    ****
ss.u41060   TCTCTGTCACAAATCCCCTTCATGAACTAAAAGCAGCTGCTTTCCCCCAGACCACTGAGA
                         190   200   210   220   230   240

                         180   190   200   210   220   230
ss.DNA164647   AAATTAGTCCGAATTGGGAATCTGGCATTAATGTTGACTTGGCAATTTCCACACGGCAAT
                    ****
ss.u41060   AAATTAGTCCGAATTGGGAATCTGGCATTAATGTTGACTTGGCAATTTCCACACGGCAAT
                         250   260   270   280   290   300

                         240   250   260   270   280   290
ss.DNA164647   ATCATCTACAACAGCTTTTCTACCGCTATGGAGAAAATAATTCTTTGTCAGTTGAAGGGT
                    ****
ss.u41060   ATCATCTACAACAGCTTTTCTACCGCTATGGAGAAAATAATTCTTTGTCAGTTGAAGGGT
                         310   320   330   340   350   360

Fig. 3A-1

```
                  300        310        320        330        340        350
ss.DNA164647  TCAGAAAATTACTTCAAAATATAGGCATAGATAAGATTAAAAGAATCCATATACACCATG
              ************************************************************
ss.u41060     TCAGAAAATTACTTCAAAATATAGGCATAGATAAGATTAAAAGAATCCATATACACCATG
                  370        380        390        400        410        420


                  360        370        380        390        400        410
ss.DNA164647  ACCACGACCATCACTCAGACCACGAGCATCACTCAGACCATGAGCGTCACTCAGACCATG
              ************************************************************
ss.u41060     ACCACGACCATCACTCAGACCACGAGCATCACTCAGACCATGAGCGTCACTCAGACCATG
                  430        440        450        460        470        480


                  420        430        440        450        460        470
ss.DNA164647  AGCATCACTCAGACCACGAGCATCACTCTGACCATGATCATCACTCCCACCATAATCATG
              ***************************************         ***********
ss.u41060     AGCATCACTCAGACCACGAGCATCACTCTG-----------------ACCATAATCATG
                  490        500        510                        520


                  480        490        500        510        520        530
ss.DNA164647  CTGCTTCTGGTAAAAATAAGCGAAAAGCTCTTTGCCCAGACCATGACTCAGATAGTTCAG
              ************************************************************
ss.u41060     CTGCTTCTGGTAAAAATAAGCGAAAAGCTCTTTGCCCAGACCATGACTCAGATAGTTCAG
                  530        540        550        560        570        580


                  540        550        560        570        580        590
ss.DNA164647  GTAAAGATCCTAGAAACAGCCAGGGGAAAGGAGCTCACCGACCAGAACATGCCAGTGGTA
              ************************************************************
ss.u41060     GTAAAGATCCTAGAAACAGCCAGGGGAAAGGAGCTCACCGACCAGAACATGCCAGTGGTA
                  590        600        610        620        630        640
```

Fig. 3A-2

```
                    600        610        620        630        640        650
ss.DNA164647   GAAGGAATGTCAAGGACAGTGTTAGTGCTAGTGAAGTGACCTCAACTGTGTACAACACTG
               ***********************************************************
ss.u41060      GAAGGAATGTCAAGGACAGTGTTAGTGCTAGTGAAGTGACCTCAACTGTGTACAACACTG
                    650        660        670        680        690        700


                    660        670        680        690        700        710
ss.DNA164647   TCTCTGAAGGAACTCACTTTCTAGAGACAATAGAGACTCCAAGACCTGGAAAACTCTTCC
               ***********************************************************
ss.u41060      TCTCTGAAGGAACTCACTTTCTAGAGACAATAGAGACTCCAAGACCTGGAAAACTCTTCC
                    710        720        730        740        750        760


                    720        730        740        750        760        770
ss.DNA164647   CCAAAGATGTAAGCAGCTCCACTCCACCCAGTGTCACATCAAAGAGCCGGGTGAGCCGGC
               ***********************************************************
ss.u41060      CCAAAGATGTAAGCAGCTCCACTCCACCCAGTGTCACATCAAAGAGCCGGGTGAGCCGGC
                    770        780        790        800        810        820


                    780        790        800        810        820        830
ss.DNA164647   TGGCTGGTAGGAAAACAAATGAATCTGTGAGTGAGCCCCGAAAGGCTTTATGTATTCCA
               ***********************************************************
ss.u41060      TGGCTGGTAGGAAAACAAATGAATCTGTGAGTGAGCCCCGAAAGGCTTTATGTATTCCA
                    830        840        850        860        870        880


                    840        850        860        870        880        890
ss.DNA164647   GAAACACAAATGAAAATCCTCAGGAGTGTTTCAATGCATCAAAGCTACTGACATCTCATG
               ***********************************************************
ss.u41060      GAAACACAAATGAAAATCCTCAGGAGTGTTTCAATGCATCAAAGCTACTGACATCTCATG
                    890        900        910        920        930        940
```

Fig. 3A-3

```
              900        910        920        930        940        950

ss.DNA164647  GCATGGGCATCCAGGTTCCGCTGAATGCAACAGAGTTCAACTATCTCTGTCCAGCCATCA
              ***********************************************************

ss.u41060     GCATGGGCATCCAGGTTCCGCTGAATGCAACAGAGTTCAACTATCTCTGTCCAGCCATCA
              950        960        970        980        990        1000


              960        970        980        990        1000       1010

ss.DNA164647  TCAACCAAATTGATGCTAGATCTTGTCTGATTCATACAAGTGAAAAGAAGGCTGAAATCC
              ***********************************************************

ss.u41060     TCAACCAAATTGATGCTAGATCTTGTCTGATTCATACAAGTGAAAAGAAGGCTGAAATCC
              1010       1020       1030       1040       1050       1060


              1020       1030       1040       1050       1060       1070

ss.DNA164647  CTCCAAAGACCTATTCATTACAAATAGCCTGGGTTGGTGGTTTTATAGCCATTTCCATCA
              ***********************************************************

ss.u41060     CTCCAAAGACCTATTCATTACAAATAGCCTGGGTTGGTGGTTTTATAGCCATTTCCATCA
              1070       1080       1090       1100       1110       1120


              1080       1090       1100       1110       1120       1130

ss.DNA164647  TCAGTTTCCTGTCTCTGCTGGGGGGTTATCTTAGTGCCTCTCATGAATCGGGTGTTTTTCA
              ***********************************************************

ss.u41060     TCAGTTTCCTGTCTCTGCTGGGGGGTTATCTTAGTGCCTCTCATGAATCGGGTGTTTTTCA
              1130       1140       1150       1160       1170       1180


              1140       1150       1160       1170       1180       1190

ss.DNA164647  AATTTCTCCTGAGTTTCCTTGTGGCACTGGCCGTTGGGACTTTGAGTGGTGATGCTTTTT
              ***********************************************************

ss.u41060     AATTTCTCCTGAGTTTCCTTGTGGCACTGGCCGTTGGGACTTTGAGTGGTGATGCTTTTT
              1190       1200       1210       1220       1230       1240
```

Fig. 3A-4

```
              1200      1210      1220      1230      1240      1250
ss.DNA164647  TACACCTTCTTCCACATTCTCATGCAAGTCACCACCATAGTCATAGCCATGAAGAACCAG
              ***********************************************************
ss.u41060     TACACCTTCTTCCACATTCTCATGCAAGTCACCACCATAGTCATAGCCATGAAGAACCAG
              1250      1260      1270      1280      1290      1300


              1260      1270      1280      1290      1300      1310
ss.DNA164647  CAATGGAAATGAAAAGAGGACCACTTTTCAGTCATCTGTCTTCTCAAAACATAGAAGAAA
              ****************************************************+*******+
ss.u41060     CAATGGAAATGAAAAGAGGACCACTTTTCAGTCATCTGTCTTCTCAAAACATAGAAGAAA
              1310      1320      1330      1340      1350      1360


              1320      1330      1340      1350      1360      1370
ss.DNA164647  GTGCCTATTTTGATTCCACGTGGAAGGGTCTAACAGCTCTAGGAGGCCTGTATTTCATGT
              ****************************************************+*+*****+
ss.u41060     GTGCCTATTTTGATTCCACGTGGAAGGGTCTAACAGCTCTAGGAGGCCTGTATTTCATGT
              1370      1380      1390      1400      1410      1420


              1380      1390      1400      1410      1420      1430
ss.DNA164647  TTCTTGTTGAACATGTCCTCACATTGATCAAACAATTTAAAGATAAGAAGAAAAAGAATC-
              *********************************+*****************-+*******+
ss.u41060     TTCTTGTTGAACATGTCCTCACATTGATCAAACAATTTAAAGATAAGAAGAAAAAGAATC
              1430      1440      1450      1460      1470      1480


              1440      1450      1460      1470      1480      1490
ss.DNA164647  AGAAGAAACCTGAAAATGATGATGATGTGGAGATTAAGAAGCAGTTGTCCAAGTATGAAT
              ***********************************************************+
ss.u41060     AGAAGAAACCTGAAAATGATGATGATGTGGAGATTAAGAAGCAGTTGTCCAAGTATGAAT
              1490      1500      1510      1520      1530      1540
```

Fig. 3A-5

```
            1500      1510      1520      1530      1540      1550
ss.DNA164647  CTCAACTTTCAACAAATGAGGAGAAAGTAGATACAGATGATCGAACTGAAGGCTATTTAC
              ***************************************************************
ss.u41060     CTCAACTTTCAACAAATGAGGAGAAAGTAGATACAGATGATCGAACTGAAGGCTATTTAC
            1550      1560      1570      1580      1590      1600


            1560      1570      1580      1590      1600      1610
ss.DNA164647  GAGCAGACTCACAAGAGCCCTCCCACTTTGATTCTCAGCAGCCTGCAGTCTTGGAAGAAG
              ***************************************************************
ss.u41060     GAGCAGACTCACAAGAGCCCTCCCACTTTGATTCTCAGCAGCCTGCAGTCTTGGAAGAAG
            1610      1620      1630      1640      1650      1660


            1620      1630      1640      1650      1660      1670
ss.DNA164647  AAGAGGTCATGATAGCTCATGCTCATCCACAGGAAGTCTACAATGAATATGTACCCAGAG
              ***************************************************************
ss.u41060     AAGAGGTCATGATAGCTCATGCTCATCCACAGGAAGTCTACAATGAATATGTACCCAGAG
            1670      1680      1690      1700      1710      1720


            1680      1690      1700      1710      1720      1730
ss.DNA164647  GGTGCAAGAATAAATGCCATTCACATTTCCACGATACACTCGGCCAGTCAGACGATCTCA
              ***************************************************************
ss.u41060     GGTGCAAGAATAAATGCCATTCACATTTCCACGATACACTCGGCCAGTCAGACGATCTCA
            1730      1740      1750      1760      1770      1780


            1740      1750      1760      1770      1780      1790
ss.DNA164647  TTCACCACCATCATGACTACCATCATATTCTCCATCATCACCACCACCAAAACCACCATC
              ***************************************************************
ss.u41060     TTCACCACCATCATGACTACCATCATATTCTCCATCATCACCACCACCAAAACCACCATC
            1790      1800      1810      1820      1830      1840
```

Fig. 3A-6

-

```
              1800       1810       1820       1830       1840       1850
ss.DNA164647  CTCACAGTCACAGCCAGCGCTACTCTCGGGAGGAGCTGAAAGATGCCGGCGTCGCCACTT
              ************************************************************
ss.u41060     CTCACAGTCACAGCCAGCGCTACTCTCGGGAGGAGCTGAAAGATGCCGGCGTCGCCACTT
              1850       1860       1870       1880       1890       1900


              1860       1870       1880       1890       1900       1910
ss.DNA164647  TGGCCTGGATGGTGATAATGGGTGATGGCCTGCACAATTTCAGCGATGGCCTAGCAATTG
              ************************************************************
ss.u41060     TGGCCTGGATGGTGATAATGGGTGATGGCCTGCACAATTTCAGCGATGGCCTAGCAATTG
              1910       1920       1930       1940       1950       1960


              1920       1930       1940       1950       1960       1970
ss.DNA164647  GTGCTGCTTTTACTGAAGGCTTATCAAGTGGTTTAAGTACTTCTGTTGCTGTGTTCTGTC
              ************************************************************
ss.u41060     GTGCTGCTTTTACTGAAGGCTTATCAAGTGGTTTAAGTACTTCTGTTGCTGTGTTCTGTC
              1970       1980       1990       2000       2010       2020


              1980       1990       2000       2010       2020       2030
ss.DNA164647  ATGAGTTGCCTCATGAATTAGGTGACTTTGCTGTTCTACTAAAGGCTGACATGACCGTTA
              *****************************************************  **********
ss.u41060     ATGAGTTGCCTCATGAATTAGGTGACTTTGCTGTTCTACTAAAGGCTGGCATGACCGTTA
              2030       2040       2050       2060       2070       2080


              2040       2050       2060       2070       2080       2090
ss.DNA164647  AGCAGGCTGTCCTTTATAATGCATTGTCAGCCATGCTGGCGTATCTTGGAATGGCAACAG
              ************************************************************
ss.u41060     AGCAGGCTGTCCTTTATAATGCATTGTCAGCCATGCTGGCGTATCTTGGAATGGCAACAG
              2090       2100       2110       2120       2130       2140
```

Fig. 3A-7

```
              2100      2110      2120      2130      2140      2150
ss.DNA164647  GAATTTTCATTGGTCATTATGCTGAAAATGTTTCTATGTGGATATTTGCACTTACTGCTG
              ************************************************************
ss.u41060     GAATTTTCATTGGTCATTATGCTGAAAATGTTTCTATGTGGATATTTGCACTTACTGCTG
              2150      2160      2170      2180      2190      2200


              2160      2170      2180      2190      2200      2210
ss.DNA164647  GCTTATTCATGTATGTTGCTCTGGTTGATATGGTACCTGAAATGCTGCACAATGATGCTA
              ************************************************************
ss.u41060     GCTTATTCATGTATGTTGCTCTGGTTGATATGGTACCTGAAATGCTGCACAATGATGCTA
              2210      2220      2230      2240      2250      2260


              2220      2230      2240      2250      2260      2270
ss.DNA164647  GTGACCATGGATGTAGCCGCTGGGGGTATTTCTTTTTACAGAATGCTGGGATGCTTTTGG
              ************************************************************
ss.u41060     GTGACCATGGATGTAGCCGCTGGGGGTATTTCTTTTTACAGAATGCTGGGATGCTTTTGG
              2270      2280      2290      2300      2310      2320


              2280      2290      2300      2310      2320      2330
ss.DNA164647  GTTTTGGAATTATGTTACTTATTTCCATATTTGAACATAAAATCGTGTTTCGTATAAATT
              *******************  ***********************  ***********
ss.u41060     GTTTTGGAATTATGTTACTTA-TTCCATATTTGAACATAAAATCGTG-TTCGTATAAATT
              2330      2340      2350      2360      2370      2380


              2340      2350      2360      2370      2380      2390
ss.DNA164647  TCTAGTTAAGGTTTAAATGCTAGAGTAGCTTAAAAAGTTGTCATAGTTTCAGTAGGTCAT
              ************************************************************
ss.u41060     TCTAGTTAAGGTTTAAATGCTAGAGTAGCTTAAAAAGTTGTCATAGTTTCAGTAGGTCAT
              2390      2400      2410      2420      2430      2440
```

Fig. 3A-8

```
                2400      2410      2420      2430      2440      2450
ss.DNA164647    AGGGAGATGAGTTTGTATGCTGTACTATGCAGCGTTTAAAGTTAGTGGGTTTTGTGATTT
                ***********************************************************
ss.u41060       AGGGAGATGAGTTTGTATGCTGTACTATGCAGCGTTTAAAGTTAGTGGGTTTTGTGATTT
                   2450      2460      2470      2480      2490      2500


                2460      2470      2480      2490      2500      2510
ss.DNA164647    TTGTATTGAATATTGCTGTCTGTTACAAAGTCAGTTAAAGGTACGTTTTAATATTTAAGT
                ********************************************************** *
ss.u41060       TTGTATTGAATATTGCTGTCTGTTACAAAGTCAGTTAAAGGTACGTTTTAATATTTAAGT
                   2510      2520      2530      2540      2550      2560


                2520      2530      2540      2550      2560      2570
ss.DNA164647    TATTCTATCTTGGAGATAAAATCTGTATGTGCAATTCACCGGTATTACCAGTTTATTATG
                ************************************************* *** *
ss.u41060       TATTCTATCTTGGAGATAAAATCTGTATGTGCAATTCACCGGTATTACCAGTTTATTATG
                   2570      2580      2590      2600      2610      2620


                2580      2590      2600      2610      2620      2630
ss.DNA164647    TAAACAAGAGATTTGGCATGACATGTTCTGTATGTTTCAGGGAAAAATGTCTTTAATGCT
                ************************************************** ** *** *
ss.u41060       TAAACAAGAGATTTGGCATGACATGTTCTGTATGTTTCAGGGAAAAATGTCTTTAATGCT
                   2630      2640      2650      2660      2670      2680


                2640      2650      2660      2670      2680      2690
ss.DNA164647    TTTTCAAGAACTAACACAGTTATTCCTATACTGGATTTTAGGTCTCTGAAGAACTGCTGG
                ***********************************************************
ss.u41060       TTTTCAAGAACTAACACAGTTATTCCTATACTGGATTTTAGGTCTCTGAAGAACTGCTGG
                   2690      2700      2710      2720      2730      2740
```

Fig. 3A-9

```
                  2700        2710        2720        2730        2740        2750

ss.DNA164647   TGTTTAGGAATAAGAATGTGCATGAAGCCTAAAATACCAAGAAAGCTTATACTGAATTTA
               ************************************************************
ss.u41060      TGTTTAGGAATAAGAATGTGCATGAAGCCTAAAATACCAAGAAAGCTTATACTGAATTTA
                  2750        2760        2770        2780        2790        2800


                  2760        2770
ss.DNA164647   AGCAAAAAAAAAAAAAAAAAAAA              (SEQ ID NO:3)
               ******  ***  *** *  *  ***
ss.u41060      AGCAAAGAAATAAAGGAGAAAAGAGAAGAATCTGAGAATTGGGGAGGCATAGATTCTTAT
                  2810        2820        2830        2840        2850        2860


ss.u41060      AAAAATCACAAAATTTGTTGTAAATTAGAGGGGAGAAATTTAGAATTAAGTATAAAAAGG
                  2870        2880        2890        2900        2910        2920


ss.u41060      CAGAATTAGTATAGAGTACATTCATTAAACATTTTTGTCAGGATTATTTCCCGTAAAAAC
                  2930        2940        2950        2960        2970        2980


ss.u41060      GTAGTGAGCACTCTCATATACTAATTAGTGTACATTTAACTTTGTATAATACAGAAATCT
                  2990        3000        3010        3020        3030        3040


ss.u41060      AAATATATTTAATGAATTCAAGCAATATACACTTGACCAAGAAATTGGAATTTCAAAATG
                  3050        3060        3070        3080        3090        3100


ss.u41060      TTCGTGCGGGTTATATACCAGATGAGTACAGTGAGTAGTTTATGTATCACCAGACTGGGT
                  3110        3120        3130        3140    .   3150        3160


ss.u41060      TATTGCCAAGTTATATATCACCAAAAGCTGTATGACTGGATGTTCTGGTTACCTGGTTTA
                  3170        3180        3190        3200        3210        3220


ss.u41060      CAAAATTATCAGAGTAGTAAAACTTTGATATATATGAGGATATTAAAACTACACTAAGTA
                  3230        3240        3250        3260        3270        3280
```

Fig. 3A-10

ss.u41060   TCATTTGATTCGATTCAGAAAGTACTTTGATATCTCTCAGTGCTTCAGTGCTATCATTGT
                3290   3300   3310   3320   3330   3340

ss.u41060   GAGCAATTGTCTTTATATACGGTACTGTAGCCATACTAGGCCTGTCTGTGGCATTCTCTA
                3350   3360   3370   3380   3390   3400

ss.u41060   GATGTTTCTTTTTTACACAATAAATTCCTTATATCAGCTTGAAAAAAAAAAAAAAAAAAA
                3410   3420   3430   3440   3450   3460

ss.u41060   A   (SEQ ID NO:1)

Fig. 3A-11

```
                10          20          30          40          50          60
pl.164647   MARKLSVILILTFALSVTNPLHELKAAAFPQTTEKISPNWESGINVDLAISTRQYHLQQL
            ************************************************************
pl.H00961   MARKLSVILILTFALSVTNPLHELKAAAFPQTTEKISPNWESGINVDLAISTRQYHLQQL
                10          20          30          40          50          60


                70          80          90          100         110         120
pl.164647   FYRYGENNSLSVEGFRKLLQNIGIDKIKRIHIHHDHDHHSDHEHHSDHERHSDHEHHSDH
            ************************************************************
pl.H00961   FYRYGENNSLSVEGFRKLLQNIGIDKIKRIHIHHDHDHHSDHEHHSDHERHSDHEHHSDH
                70          80          90          100         110         120


                130         140         150         160         170         180
pl.164647   EHHSDHDHHSHHNHAASGKNKRKALCPDHDSDSSGKDPRNSQGKGAHRPEHASGRRNVKD
            *****       **********************************************
pl.H00961   EHHSD------HNHAASGKNKRKALCPDHDSDSSGKDPRNSQGKGAHRPEHASGRRNVKD
                        130         140         150         160         170


                190         200         210         220         230         240
pl.164647   SVSASEVTSTVYNTVSEGTHFLETIETPRPGKLFPKDVSSSTPPSVTSKSRVSRLAGRKT
            ************************************************************
pl.H00961   SVSASEVTSTVYNTVSEGTHFLETIETPRPGKLFPKDVSSSTPPSVTSKSRVSRLAGRKT
                180         190         200         210         220         230


                250         260         270         280        · 290         300
pl.164647   NESVSEPRKGFMYSRNTNENPQECFNASKLLTSHGMGIQVPLNATEFNYLCPAIINQIDA
            ************************************************************
pl.H00961   NESVSEPRKGFMYSRNTNENPQECFNASKLLTSHGMGIQVPLNATEFNYLCPAIINQIDA
                240         250         260         270         280         290
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Fig. 4-1

```
                 310       320       330       340       350       360
pl.164647   RSCLIHTSEKKAEIPPKTYSLQIAWVGGFIAISIISFLSLLGVILVPLMNRVFFKFLLSF
            ************************************************************
pl.H00961   RSCLIHTSEKKAEIPPKTYSLQIAWVGGFIAISIISFLSLLGVILVPLMNRVFFKFLLSF
                 300       310       320       330       340       350


                 370       380       390       400       410       420
pl.164647   LVALAVGTLSGDAFLHLLPHSHASHHHSHSHEEPAMEMKRGPLFSHLSSQNIEESAYFDS
            ************************************************************
pl.H00961   LVALAVGTLSGDAFLHLLPHSHASHHHSHSHEEPAMEMKRGPLFSHLSSQNIEESAYFDS
                 360       370       380       390       400       410


                 430       440       450       460       470       480
pl.164647   TWKGLTALGGLYFMFLVEHVLTLIKQFKDKKKKNQKKPENDDDVEIKKQLSKYESQLSTN
            ************************************************************
pl.H00961   TWKGLTALGGLYFMFLVEHVLTLIKQFKDKKKKNQKKPENDDDVEIKKQLSKYESQLSTN
                 420       430       440       450       460       470


                 490       500       510       520       530       540
pl.164647   EEKVDTDDRTEGYLRADSQEPSHFDSQQPAVLEEEEVMIAHAHPQEVYNEYVPRGCKNKC    . .
            ************************************************************
pl.H00961   EEKVDTDDRTEGYLRADSQEPSHFDSQQPAVLEEEEVMIAHAHPQEVYNEYVPRGCKNKC
                 480       490       500       510       520       530


                 550       560       570       580       590       600
pl.164647   HSHFHDTLGQSDDLIHHHHDYHHILHHHHHQNHHPHSHSQRYSREELKDAGVATLAWMVI
            ************************************************************
pl.H00961   HSHFHDTLGQSDDLIHHHHDYHHILHHHHHQNHHPHSHSQRYSREELKDAGVATLAWMVI
                 540       550       560       570       580       590
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Fig. 4-2

```
                 610        620        630        640        650        660
p1.164647   MGDGLHNFSDGLAIGAAFTEGLSSGLSTSVAVFCHELPHELGDFAVLLKADMTVKQAVLY
            **********************************************.*********
p1.H00961   MGDGLHNFSDGLAIGAAFTEGLSSGLSTSVAVFCHELPHELGDFAVLLKAGMTVKQAVLY
                 600        610        620        630        640        650


                 670        680        690        700        710        720
p1.164647   NALSAMLAYLGMATGIFIGHYAENVSMWIFALTAGLFMYVALVDMVPEMLHHDASDHGCS
            *********************************************************
p1.H00961   NALSAMLAYLGMATGIFIGHYAENVSMWIFALTAGLFMYVALVDMVPEMLHNDASDHGCS
                 660        670        680        690        700        710


                 730        740        750
p1.164647   RWGYFFLQNAGMLLGFGIMLLISIFEHKIVFRINF              (SEQ ID NO:4)
            *********************.  .. *     .*
p1.H00961   RWGYFFLQNAGMLLGFGIMLLIPYLNIKSC-SYKFLVKV           (SEQ ID NO:2)
                 720        730        740        750
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Fig. 4-3

Fig. 5
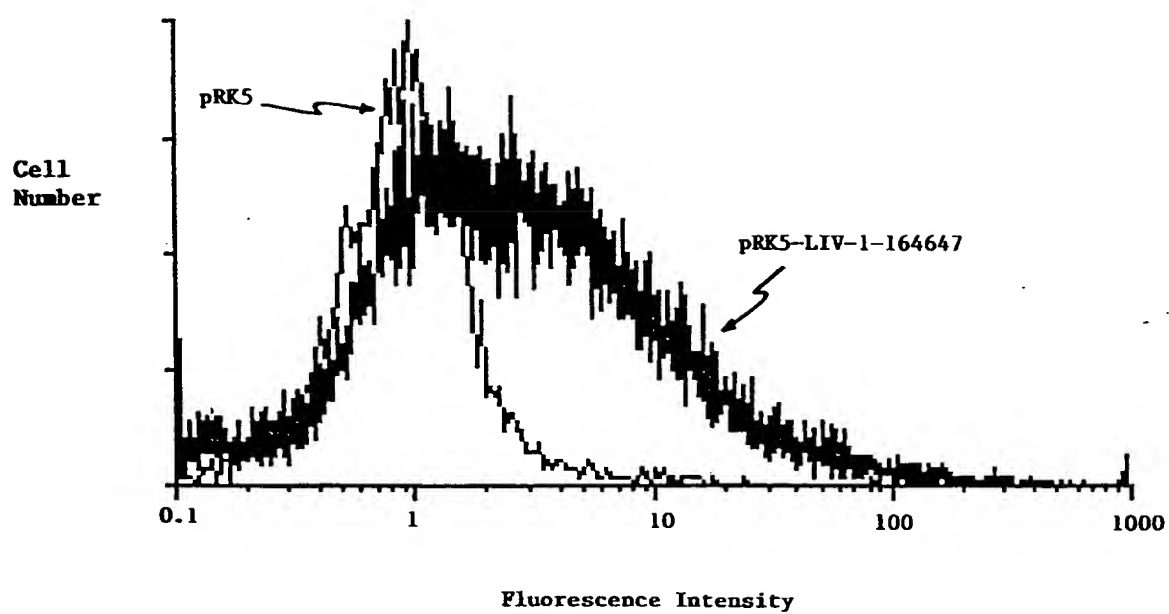
Fig. 6
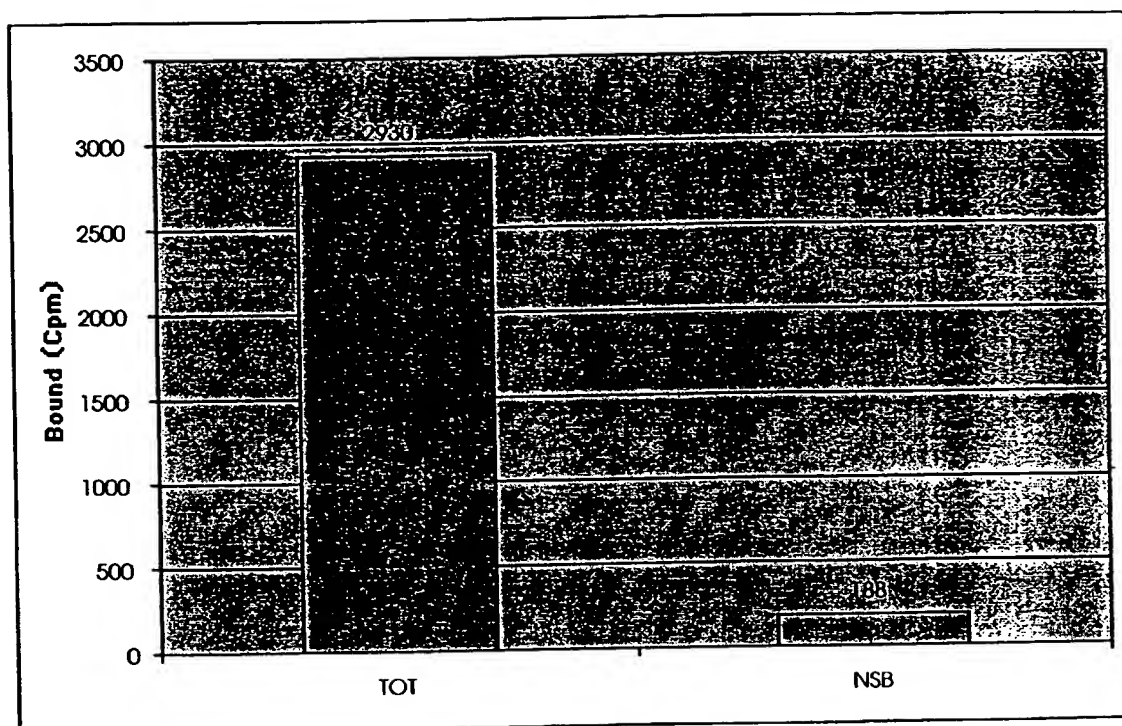
Fig. 7

Sequence Listing

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aagaatccat atacaccatg accacgacca tcactcagac cacgagcatc 450

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tctttgccca gaccatgact cagatagttc aggtaaagat cctagaaaca 600

gccaggggaa aggagctcac cgaccagaac atgccagtgg tagaaggaat 650

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      Arg Lys Ala Leu Cys Pro Asp His Asp Ser Asp Ser Ser Gly Lys
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      Ala Ser Gly Arg Arg Asn Val Lys Asp Ser Val Ser Ala Ser Glu
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      Pro Arg Lys Gly Phe Met Tyr Ser Arg Asn Thr Asn Glu Asn Pro
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      Gln Glu Cys Phe Asn Ala Ser Lys Leu Leu Thr Ser His Gly Met
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      Gln Ile Ala Trp Val Gly Gly Phe Ile Ala Ile Ser Ile Ile Ser
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       Lys Tyr Glu Ser Gln Leu Ser Thr Asn Glu Glu Lys Val Asp Thr
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       Asp Asp Arg Thr Glu Gly Tyr Leu Arg Ala Asp Ser Gln Glu Pro
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       Ser His Phe Asp Ser Gln Gln Pro Ala Val Leu Glu Glu Glu Glu
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       Val Pro Arg Gly Cys Lys Asn Lys Cys His Ser His Phe His Asp
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       His His Ile Leu His His His His His Gln Asn His His Pro His
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Gln Thr Thr Glu Lys Ile Ser Pro Asn Trp Glu Ser Gly Ile Asn
                 35                  40                  45

Val Asp Leu Ala Ile Ser Thr Arg Gln Tyr His Leu Gln Gln Leu
                 50                  55                  60

Phe Tyr Arg Tyr Gly Glu Asn Asn Ser Leu Ser Val Glu Gly Phe
                 65                  70                  75

Arg Lys Leu Leu Gln Asn Ile Gly Ile Asp Lys Ile Lys Arg Ile
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His Ile His His Asp His Asp His His Ser Asp His Glu His His
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Ser Asp His Glu Arg His Ser Asp His Glu His His Ser Asp His
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Glu His His Ser Asp His Asp His His Ser His His Asn His Ala
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Ala Ser Gly Lys Asn Lys Arg Lys Ala Leu Cys Pro Asp His Asp
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Ser Asp Ser Ser Gly Lys Asp Pro Arg Asn Ser Gln Gly Lys Gly
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Ser Val Ser Ala Ser Glu Val Thr Ser Thr Val Tyr Asn Thr Val
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Gly Lys Leu Phe Pro Lys Asp Val Ser Ser Ser Thr Pro Pro Ser
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Val Thr Ser Lys Ser Arg Val Ser Arg Leu Ala Gly Arg Lys Thr
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Asn Glu Ser Val Ser Glu Pro Arg Lys Gly Phe Met Tyr Ser Arg
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                      320                 325                     330
 15
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      Leu Val Glu His Val Leu Thr Leu Ile Lys Gln Phe Lys Asp Lys
                      440                 445                     450

 40   Lys Lys Lys Asn Gln Lys Lys Pro Glu Asn Asp Asp Asp Val Glu
                      455                 460                     465

      Ile Lys Lys Gln Leu Ser Lys Tyr Glu Ser Gln Leu Ser Thr Asn
                      470                 475                     480
 45
      Glu Glu Lys Val Asp Thr Asp Asp Arg Thr Glu Gly Tyr Leu Arg
                      485                 490                     495

      Ala Asp Ser Gln Glu Pro Ser His Phe Asp Ser Gln Gln Pro Ala
 50                 500                 505                     510

      Val Leu Glu Glu Glu Glu Val Met Ile Ala His Ala His Pro Gln
                      515                 520                     525

 55     Glu Val Tyr Asn Glu Tyr Val Pro Arg Gly Cys Lys Asn Lys Cys
                      530                 535                     540

      His Ser His Phe His Asp Thr Leu Gly Gln Ser Asp Asp Leu Ile
                      545                 550                     555
 60
      His His His His Asp Tyr His His Ile Leu His His His His His
                      560                 565                     570
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9

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          Gln Asn His His Pro His Ser His Ser Gln Arg Tyr Ser Arg Glu
                          575             580                 585

          Glu Leu Lys Asp Ala Gly Val Ala Thr Leu Ala Trp Met Val Ile
 5                        590             595                 600

          Met Gly Asp Gly Leu His Asn Phe Ser Asp Gly Leu Ala Ile Gly
                          605             610                 615

10        Ala Ala Phe Thr Glu Gly Leu Ser Ser Gly Leu Ser Thr Ser Val
                          620             625                 630

          Ala Val Phe Cys His Glu Leu Pro His Glu Leu Gly Asp Phe Ala
                          635             640                 645
15
          Val Leu Leu Lys Ala (Asp) Met Thr Val Lys Gln Ala Val Leu Tyr
                          650             655                 660

          Asn Ala Leu Ser Ala Met Leu Ala Tyr Leu Gly Met Ala Thr Gly
20                        665             670                 675

          Ile Phe Ile Gly His Tyr Ala Glu Asn Val Ser Met Trp Ile Phe
                          680             685                 690

25        Ala Leu Thr Ala Gly Leu Phe Met Tyr Val Ala Leu Val Asp Met
                          695             700                 705

          Val Pro Glu Met Leu His Asn Asp Ala Ser Asp His Gly Cys Ser
                          710             715                 720
30
          Arg Trp Gly Tyr Phe Phe Leu Gln Asn Ala Gly Met Leu Leu Gly
                          725             730                 735

          Phe Gly Ile Met Leu Leu Ile Ser Ile Phe Glu His Lys Ile Val
35                        740             745                 750

          Phe Arg Ile Asn Phe
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      <221> Unknown
50   <222> 245, 308, 313, 352
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       tgctcacaat gatagcactg aagcactgag agatatcaaa gtactttctg 150

60   aatcgaatca aatgatactt agtgtagttt taatatcctc atatatatca 200

       aagttttact actctgataa ttttgtaaac cagggtaacc aggancatcc 250

agtcatacag cttttgggtg atatataact tgggcaataa cccagtctgg 300

gtgatacnta aanctactca ctgtactcat ctgggtatat acccgcacgg 350

ancattttgg aaattcccaa tttcttgggt caggtgatat a 391

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<210> 7
<211> 27
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<210> 9
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<211> 43
<212> DNA
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<220>
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5    <220>
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10    1               5                   10                  15

     His Gln


15   <210> 12
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     accatcctca cagtcacagc cagcgctact ctcgggagga gctgaaagat 150

30   gccggcgtcg ccactttggc ctggatggtg ataatgggtg atggcctgca 200

     caatttcagc gatggcctag caattggtgc tgcttttact gaaggcttat 250

     caagtggttt aagtacttct gttgctgtgt tctgtcatga gttgcctcat 300
35
     gaattaggtg actttgctgt tctactaaag gctgacatga ccgttaagca 350

     ggctgtcctt tataatgcat tgtcagccat gctggcgtat cttggaatgg 400

40   caacaggaat tttcattggt cattatgctg aaaatgtttc tatgtggata 450

     tttgcactta ctgctggctt attcatgtat gttgctctgg ttgatatggt 500

     acctgaaatg ctgcacaatg atgctagtga ccatggatgt agccgctggg 550
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     g 551

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ggtggagccg ctgacaccta gcggagcgat gcccaaccag gcgcagatgc  150

ggatcctgaa agagacggag ctgaggaagg tgaaggtgct tggatctggc  200

gcttttggca cagtctacaa gggcatctgg atccctgatg gggagaatgt  250

gaaaattcca gtggccatca aagtgttgag ggaaaacaca tcccccaaag  300

ccaacaaaga aatcttagac gaagcatacg tgatggctgg tgtgggctcc  350

ccatatgtct cccgccttct gggcatctgc ctgacatcca cggtgcagct  400

ggtgacacag cttatgccct atggctgcct cttagaccat gtccgggaaa  450

accgcggacg cctgggctcc caggacctgc tgaactggtg tatgcagatt  500

gccaagggga tgagctacct ggaggatgtg cggctcgtac acagggactt  550

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cacgaaacta ccttcaactc catcatgaag tgtgactgtg acatccgcaa  150

agacctgtac gccaacacag tgctgtctgg cggcaccacc atgtaccctg  200

gcattgccga caggatgcag aaggagatca ctgccctggc acccagcaca  250

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1   5

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1   5   10

<210> 19
<211> 22
<212> PRT
<213> Homo sapien

<400> 19
His Glu His His Ser Asp His Glu His His Ser Asp His Asp His
1   5   10   15

His Ser His His Asn His Ala
                20